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NATIONAL CANCER INSTITUTE

ANNUAL REPORT

October 1, 1977 through September 30, 1978

Part II-A

DIVISION OF CANCER BIOLOGY AND DIAGNOSIS

U.S. NATIONAL CANCER INSTITUTE
REPORT OF PROGRAM ACTIVITIES
ANNUAL REPORT

October 1, 1977 through September 30, 1978

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DIVISION OF CANCER BIOLOGY AND DIAGNOSIS
NATIONAL CANCER INSTITUTE

SUMMARY REPORT
OF THE
DIRECTOR

October 1, 1977 Through September 30, 1978

INTRODUCTION

The Division of Cancer Biology and Diagnosis consists of both intramural and collaborative research programs in the biology and diagnosis of cancer. The Division also coordinates the activities of the Breast Cancer Task Force and the Lung Cancer Program, and manages a collaborative program in immunology. A close coordination is maintained with other divisions of NCI in areas of common interest.

The intramural research program consists of three broad areas: biology, immunology, and clinical research. A group of laboratories performing basic research in cancer biology includes the Laboratories of Biochemistry, Pathophysiology, Molecular Biology, and Theoretical Biology. Four units, Immunology Branch and the Laboratories of Immunobiology, Immunodiagnosis and Cell Biology, together with a sizeable contract program, are assigned to the Associate Director for Immunology. Three clinical branches work in the fields of Dermatology, Metabolism, and Pathology. A fourth organizational element, under the Associate Director for Extramural Research, manages the three major contract programs: Cancer Diagnosis, Breast Cancer Task Force, and Cytology Automation.

Except for personnel shifts arising from retirements and reassignments, no major organizational changes have occurred last year.

Much of the research in cancer biology--other than tumor immunology and clinical studies--is conducted in the laboratories of Biochemistry, Molecular Biology, Pathophysiology and Theoretical Biology. Only a few contracts support this almost entirely intramural program.

The Laboratory of Biochemistry, directed by Dr. Robert F. Goldberger, was divided into six sections. The new P3 facility was used extensively this year for molecular cloning studies.

The control of gene expression and its application to clinical medicine was the domain of the Biochemistry of Gene Expression Section. Work centered on the effects of steroid hormones on enzyme induction and cell function in tissue culture, and on steroid receptors in cells from leukemia and breast cancer patients. The steroid receptors and the responses in functional subsets of T lymphoblasts were defined. Data from breast cancer patients showed that lack of estrogen receptors correlates with a high response rate to chemotherapy; presence of the receptors is associated with a poor response.

The Biosynthesis Section concentrated on the relationship between cell function and organization at the molecular level. The enzymatic aspects of DNA and collagen biosynthesis were investigated as well as the regulation and functional correlates of endogenous retrovirus expression in normal and transformed cells. Treating cells with local anesthetics was found to inhibit collagen secretion as much as 98%. In another area, several mouse myeloma proteins important for analysis of in vitro DNA replication were purified to near homogeneity. An unusually large 60-fold induction of α-polymerase activity was obtained when monkey cells were infected with adenovirus 2.

The Cellular Regulation Section studied the regulation of gene expression at the molecular level, focusing on the way estrogen induces synthesis of egg yolk phosphoprotein in rooster liver, and in particular on the molecules involved in the transcription of the phosphoprotein gene. In one study, a fraction of chicken repetitive DNA which represents sequences conserved during at least 60 million years of avian evolution was obtained. Using a new process, DNA-dependent RNA polymerase II was isolated from rooster liver, which was also found to contain about 14,000 different species of mRNA. A procedure was developed for isolating rooster liver nuclei active in transcription; nuclei from estrogen-treated liver were about twice as active as untreated ones.

The Developmental Biochemistry Section examined the structure and transcription of the eukaryote genome in studies on frog mitochondrial DNA, Drosophila ribosomal DNA, the gene coding for frog yolk proteins and the pattern of gene expression in embryos.

Work in the Nucleic Acid Enzymology Section concerned the structure and metabolism of nucleic acids and nucleotides. The role of calcium-dependent regulator protein in regulating cyclic AMP levels and the phosphorylation of myosin light chains was studied. Other systems under investigation included the organization of the monkey genome and the structure of defective variants of simian virus 40 (SV40). Nucleotide sequences of two variants of SV40 were almost completely determined.

The Protein Chemistry Section explored the physical and chemical aspects of proteins involved in the synthesizing and differentiating systems of organisms. Studies in this project included transfer of isolated chromosomes to mammalian cells, granulocyte development, fractionation of cells involved in cellular immune reactions and protein fractionation techniques.

Under the direction of Dr. Ira Pastan, the Laboratory of Molecular Biology continued investigations into six major areas.

Several projects were aimed at identifying the viral gene products which initiate cell transformation and at determining how these products affect cyclic AMP, cell surface protein (CSP) and collagen synthesis. A very unusual protein, p. 56, with a half-life of less than 30 minutes, was identified and appears to be the product of the transforming gene of Rous Sarcoma Virus (RSV). Another study has shown that RSV transformation decreases synthesis of CSP and collagen by lowering the cellular content of translatable mRNA for these molecules. A protein which transformed cells excrete in large quantities was identified and purified to homogeneity.

Development of two new techniques provided insights into the factors controlling cell shape and movement. Using electron microscopy, labelled antibodies were localized with good morphologic preservation. A video camera with a low light level intensifier and taping system made it possible to see small amounts of fluorescently-labeled proteins in living cells. With these techniques, it was shown that molecules such as α_2 macroglobulin, insulin and epidermal growth factor first bind to cell surfaces diffusely, and later accumulate into patches.

Studies regarding the regulation of gene expression emphasized identification of molecules which activate genes and determination of the structure and function of the regulatory regions of genes. The overlapping promoters, P1 and P2, were found to regulate the expression of the *gal* operon of *E. coli*. It was shown that the cyclic AMP receptor (CRP) and cyclic AMP activate only P1, while P2 is repressed by CRP and is probably subject to another regulatory mechanism. The nucleotide sequence of the entire regulatory region for wild type *gal* operon and the base changes in several mutants in the region were determined.

In the area of recombination and DNA synthesis, several DNA replication proteins of *E. coli* were purified and studied. One of these, the *dnaG* protein, was found to catalyze the synthesis of sequence specific oligonucleotides which serve as primers for DNA elongation. Six other proteins function prior to

dnaG activity, presumably by creating a dnaG recognition site. Interactions of these proteins with each other, with nucleotides, and with DNA were investigated.

Studies of the thyroid cell were aimed at understanding how it forms, stores and releases its hormone. Factors which control the growth, differentiation and malignant transformation of the thyroid cell were examined as well. For example, some types of cells recently recognized as present in the normal thyroid gland were studied. These may be the cells of origin of some previously observed histologic tumors.

Another area of inquiry concerned how the mammary tumor virus and other oncornaviruses are synthesized and how they change normal cells into cancer cells.

A wide range of studies on the basic development of normal and neoplastic cells was continued by the Laboratory of Pathophysiology, under the direction of Dr. Pietro Gullino.

One concern in the area of mammary tissue pathophysiology was the mechanism of hormone-dependent mammary tumor regression. Injection of dibutyryl cyclic AMP (DBcAMP)into tumor-bearing hosts was found to produce growth arrest and regression in several hormone-dependent and hormone-independent tumors. The tumor regression induced by either DBcAMP treatment or ovariectomy was found to occur through a similar biochemical mechanism. The regression which followed both treatments was characterized by three events: decrease of estrogen receptors, increase of cAMP receptors and phosphorylation of an endogenous nuclear protein. Estrogen receptors in several experimental carcinomas were studied in terms of tumor regression and receptor translocation to the nucleus. Possible clinical use of the ratio of estrogen to cAMP receptors as a marker of hormone responsiveness in mammary carcinoma was investigated.

The regulatory mechanism of milk protein production in normal and neoplastic mammary epithelium was studied. The characteristics of two species of α-lactalbumin (α-LA) previously isolated in rat milk were further described, purification of rat casein was completed and regulation of α-LA production under pituitary stimulation was shown to vary among different mammary carcinomas.

In a related area, cultures of mouse mammary glands were used to study how thyroid hormones act on the mammary gland. It was demonstrated that DNA synthesis in vitro is not necessary if the mammary gland of virgins is stimulated in vivo by thyroxine before explanting. When milk proteins were induced in vitro, prostaglandin F2α(like thyroid hormones) overcame the need for DNA synthesis and enhanced the production of αLA. The specific binding proteins for LT3 were identified in both the nuclei and cytoplasms of epithelium from euthyroid glands.

The role of poly(ADP-Ribose) in initiating cell division was probed further, with the focus on the interaction of poly(ADP-Ribose) and the basic

chromosomal proteins (particularly histone H1) with which it is associated. It was observed that: (1) Chromatin-bound poly(ADP-Ribose) polymerase attaches polymer chains to histone H1, producing a complex of two H1 molecules cross-linked by one polymer chain. (2) The polymer is in a protected environment within the complex. (3) The H1+ polymer complex binds to DNA and exposes the polymer. (4) Conformational changes of chromatin produced by polyamines selectively promote formation of the H1+poly(ADP-Ribose) complex. It was hypothesized that poly(ADP-Ribose) has a role in modifying the structure of chromosomal fiber by serving as a bridge between proximal folds of the fiber, thus acting as a local, transient fixative. Such a process appears to be essential to cell division, for arrest of polymer synthesis produces cell death.

The involvement of ribosomal subunits and mRNA in the onset and arrest of lymphocyte growth was examined. In lymphocytes, addition of antomycin D was shown to cause decreased protein synthesis by acting on the nucleus. Although resting lymphocytes gradually lost the ability to synthesize protein, they very quickly failed to be stimulated by mitogens. The possibility that some form of RNA could be responsible for limiting the rate of protein synthesis in resting lymphocytes was investigated. A powerful ribonuclease inhibitor which protects ribonucleoproteins during cell lysis and purification was synthesized.

Research continued on the structure and functions of biological membranes, in order to better understand cell-to-cell interaction, and to provide a basis for comprehending the mechanism of metastasis.

Another area of interest concerned the properties and biological significance of extranuclear DNA. Difficulties in differentiating various kinds of DNA present in the cytoplasm were mitigated when cetyl trimethylammonium bromide was used to precipitate the DNA from tissue samples. In rat liver, microsomes and mitochondria were each found to contain about 1% of the total liver DNA.

The causes of cancer cachexia were studied with a view toward blocking and reversing these systemic effects of cancer so that cancer patients could be better prepared for anti-cancer therapies. Work centered on total energy, water and other material exchanges and on the physiological and behavioral control of food and water intake in normal and cancerous organisms. Some of the components of normal feeding control which are impaired (and unimpaired) by tumor growth were established, and these may have a synergistic effect on food intake.

Another project area looked at the effects of ionizing radiation on proteins, DNA and bone marrow cells. The "quality" of radiation was discovered to play a major role in conditioning the survival of bone marrow cell populations.

Much of the work of the Laboratory of Theoretical Biology, directed by Dr. Mones Berman, was related to mathematical modeling, but some experiments were carried out in laboratories. Much of the theoretical work was done in cooperation with other groups.

The research on membranes included studies of directed interaction between liposomes and cells and possible clinical uses of liposomes. A novel approach was developed to obtain preferential delivery of liposome-encapsulated drugs to certain areas--to tumors and sites of infection, for example. Liposomes were designed to release drugs primarily in areas heated a few degrees above physiological. In the presence of serum, drug release at 43^{o} could be made 100 times greater than that at 37^{o}.

A mathematical model was developed to explain the biochemical pathway chosen by the cell in terms of the physical chemistry of cell surface events. The theory also provided an explanation for the wide range of qualitative differences observed in dose response curves.

In the area of theoretical immunology, a mathematical model was used to describe characteristics of cyclical antibody production and to make several predictions, including the effects of the timing and magnitude of passive antibody administration and the existence of an optimal antigenic dose. It also predicted something completely new: cyclic variations in antibody affinity.

Development of models of the endocrine and other dynamic systems continued. Data from experiments in man were used to create kinetic models of plasma apoproteins, cholesterol and triglyceride. Synthesis pathways for bile cholesterol and bile acids were identified and measured in T-tube patients, indicating that most of the newly synthesized cholesterol cycles through the plasma and that about 75% of the bile secretions are supplied from plasma precursors.

Kinetic models of the glucose regulatory systems were formulated for man and other animals. These included models of the distribution, metabolism and receptor binding of insulin and models of the role of insulin in the control of glucose utilization and production. The first detailed model which interrelates glucose, alanine and lactate carbons was formulated. The model demonstrated that alanine (and probably lactate) carbons do not behave homogeneously and showed that this heterogeneity must be dealt with in order to calculate correctly the rates of interconversion and synthesis. Obesity, diabetes, aging, and the cachexia of advanced cancer are examples of metabolic conditions in which glucose homeostasis is perturbed and which could therefore be better understood by use of these models.

Work has continued on devising the SAAM computer system for the simulation, analysis and modeling of biokinetic systems. The computational power of SAAM was increased, and new conversational commands made it easier to use. This program has been widely distributed and staff of the Laboratory of Theoretical Biology assisted investigators who use it.

The immunology program includes four units under the Associate Director for Immunology: the Immunology Branch and the Laboratories of Immunodiagnosis, Immunobiology and Cell Biology. Although the Metabolism Branch is part of the Clinical Research program, its progress is discussed here because much of its work is closer to that of immunology.

The Immunology Branch, under the direction of Dr. William Terry, carried out laboratory studies of regulation and control of immune responses, structure and function of cell surfaces, transplantation biology and tumor immunology. Clinical studies relevant to tumor immunology were also performed.

The development of an *in vitro* system for generating primary and secondary antibody responses to soluble antigens facilitated study of the genetic control of cellular interactions involved in these responses. The generation of specific antibody responses required the active recognition by T cells of H-2 antigens (probably Ia antigens) as "self" markers which are expressed by accessory cells. The H-2 antigens recognized as "self" were shown to be determined by the genotype of the thymus in which the T cells mature, rather than by the genotype of the T cells.

In vivo studies of genetic control used the complex antigen staphylococcal nuclease and idiotypic determinants produced in rats. After screening a variety of strains and offspring, it was shown that idiotype expression is linked to the heavy chain allotype marker, rather than to the immune response gene locus. Results also indicated that H-2-linked immune response gene control must have an effect at both the B cell and the T cell level.

In studying the functional and serologic characteristics of cells important to the immune response, the expression of Ia antigens on functional cell populations has been examined. Anti-Ia plus complement was found to inactivate a glass-adherent non-T, non-B radioresistant accessory cell which is required for the induction of a proliferative response to concanavallin A. Genetic studies showed that the Ia determinants are encoded by at least two subregions of the I locus.

Among projects studying the structure and function of cell surfaces were studies of cell membrane damage caused by immunologic mechanisms. The damage caused by antibody plus complement was associated with complete loss of membrane potential and electrical resistance. This damage occurred within 1-2 minutes after adding the complement to the system, but could be reversed by removing the complement.

Research related to transplantation was aimed at understanding the role of the products of the major histocompatibility complex in the cellular interactions of the immune response. Three herds of miniature swine have been developed, each homozygous for a different set of histocompatibility antigens. Renal allografts within one herd appeared to survive indefinitely despite major antigenic differences. Rejection, however, occurred within

the other two herds, with variable kidney survival times. In humans, antigens associated with the major histocompatibility complex have been linked with a variety of diseases. Patients suffering from acute leukemia and acute schizophrenia have been found to have high levels of HLA-A2.

Tumor immunology projects included a controlled, randomized trial comparing immunotherapy to chemotherapy in patients with malignant melanoma. It is too early to evaluate the trial, but thus far no striking differences have been noted between the groups of patients. Preliminary results, however, suggest there may be a psychological variable as useful as some biological factors currently employed to predict short-term prognosis for this tumor.

The three sections of the Laboratory of Immunobiology, headed by Dr. Herbert Rapp, sought methods of killing tumor cells *in vitro*, eradicating tumors and metastases *in vivo*, and overcoming possible suppression of host immunity by tumors.

In the Cellular Immunity Section, a guinea pig hepatoma model was used to test immunological treatment of post-surgical malignancies. Intralesionally injected BCG cell walls incorporated into mineral oil droplets was discovered to be at least as effective as living BCG in eradicating tumors and microscopic lymph node metastases, and it lacked the toxic side-effects of living BCG. Emulsions of BCG cell walls were found to be about as effective as local surgery in controlling naturally occurring bovine squamous cell carcinoma. Tests with mice and guinea pigs showed that BCG cell wall skeletons, MER, cord factor, a synthetic analog of cord factor and sulfolipids also have anti-tumor activity when injected intralesionally in oil/water emulsion.

Guinea pigs with transplantable hepatocellular carcinoma were used to evaluate the influence of tumor dose, tumor burden and timing of surgery on the development of resistance to tumor challenge. When tumors were removed 7 days after and metastases were removed 21 days after implantation, animals failed to reject a challenge with 10^6 viable line 10 cells. Animals whose tumors and metastases were not surgically removed until 21 days after implantation, however, put up strong resistance to tumor challenge.

Efforts continued to develop ways of eradicating malignant disease remaining after surgery or other treatment. Guinea pigs with established dermal tumors and microscopic metastases were treated by excision of tumors followed by immunization with various vaccines with or without added tumor cells. Vaccines containing adjuvants and tumor cells prevented the growth of lymph node metastases in a significant number of animals.

The Humoral Immunity Section was concerned with the cellular events which render tumor cells either resistant or susceptible to killing by antibody and complement. Studies continued of how enzymes, metabolic inhibitors and anti-lipidemic agents make tumor cells more sensitive to immune attack, and how hormones decrease this sensitivity. The ability of tumor cells to resist humoral immune attack was correlated with their ability to synthesize complex lipids, but not with synthesis of DNA, RNA, protein or complex carbohydrate. Hormone-induced increase in tumor cells'

resistance to killing was accompanied by an enhancement in the synthesis of certain complex lipids, specifically, phospholipids and triglycerides.

Studies of the interaction of antibody and complement determined the molecular formulas for complexes formed between Protein A and IgG antibodies; the largest of these complexes behaved in some ways like IgM. An offshoot of this research was the creation of a general immunoassay method which circumvents the need for labelled antigen or antibody.

In studying the interaction of host and tumor, the Immunopathology Section looked closely at the role of macrophages. Activated macrophages--those with direct tumor-killing ability--develop during a variety of immune reactions if an effective activation stimulus is present. Work was aimed at characterizing the lymphocyte derived factors (lymphokines) required for macrophage activation. Evidence thus far indicates that the precursors of macrophages activated by lymphokines are immature blood-derived mononuclear phagocytes. Mice genetically incapable of developing tumor-killing macrophages in response to certain stimuli were used to study the pathways for macrophage activation. Macrophages from such mice with transplanted syngeneic fibrosarcomas were found to have depressed chemotactic responses and markedly enhanced phagocytosis. This enhanced phagocytosis, but not the chemotactic defect, could be transferred to normal mice by serum. Characterization was begun of a serum factor, macrophage stimulating protein (MSP), found to promote chemotaxis.

In another project, a molecule which increases basophil chemotaxis response to a complement-derived or leukocyte-derived chemotactic factor was isolated from phytohemagglutinin. Efforts to use the release of tritium from labelled target cells as an assay of lymphotoxin have also continued.

The research program of the Laboratory of Immunodiagnosis, directed by Dr. Ronald Herberman, was divided into three parts: cell-mediated immunity studies in animal tumor systems, in humans, and immunochemical studies of tumor-associated antigens (TAA). In all areas, emphasis was placed on applying research information to diagnosing and monitoring cancer.

Extensive efforts were made to characterize natural killer (NK) cells in mice, rats and humans. Inoculation of viruses, immune adjuvants, interferon inducers and interferon all boosted NK activity. Interferon was shown to have a central role in activating NK cells. However, the *in vitro* effects of interferon or poly I:C could be blocked by adding anti-interferon. The NK response to poly I:C appeared to be mediated by the macrophage-dependent production of interferon; this mechanism seemed to explain the inhibitory effects of carrageenan and silica on NK activity. Experiments with immunopharmacologic agents indicated that NK cells may develop from precursors which are substantially more resistant to X-irradiation and cytologic drugs.

The anti-tumor effects of macrophages and their ability to help or suppress immune responses were investigated. Cytologic macrophages recovered from the tumors of mice injected 14 days earlier with either

regressor or progressor stock of murine sarcoma virus (MSV) had comparable cytotoxicity levels. However, macrophages from more progressed tumors--at 50 to 60 days after inoculation--had lower levels of activity. Nude mice were found not to have cytologic macrophages in their tumors. LPS was found to increase macrophage-mediated cytotoxicity, but it enhanced the activity of only those cells which were already reactive.

By means of an anti-T cell serum, the T cell lineage of human NK and K cells was confirmed. The NK cell was found to be an FcR+ lymphocyte of T cell lineage inseparable from and perhaps identical to the killer (K) cell that mediates ADCC in the Chang liver cell system. Preexisting NK and K cell activities---as shown with mouse NK cells and interferon--could be boosted by purified interferon preparations from lymphoblastoid cell lines, fibroblasts or lymphocytes. Interferon did not facilitate development of FcR- precursor cells into FcR+ effector cells. Recent data from patients receiving poly I:C-polylysine complex indicated that levels of NK and ADCC activities are boosted at 2 days.

Cellular immunity to tumor antigens was evaluated and monitored using microassays of direct and indirect migration inhibition. Spleen cells from C3H+ mice responded to mouse mammary tumor virus (MuMTV) and produced a factor--presumably the macrophage migration inhibition factor, MIF--which inhibited migration of normal mouse peritoneal exudate cells. In contrast, spleen cells from C3H- mice did not display reactivity. Normal mice developed migration inhibition reactivity to MuMTV if housed in the same room as MuMTV-bearing mice, suggesting horizontal transmission of the virus.

Direct macrophage migration inhibition micro-assays were used to monitor TAA activity of SV-40 virus-induced tumors, plasmacytomas and Meth-A tumors. The antigen preparations included cell-free crude membranes, papain-solubilized and NP40 detergent-solubilized membrane extracts. This extremely sensitive assay could detect migration inhibition reactivity at picogram concentrations of all three types of antigenic preparations.

Studies of the cellular immune competence of cancer patients continued. The mononuclear peripheral blood leukocytes (MPBL) of lung and breast cancer patients who showed decreased proliferative responses to PHA, Con A and MLC were analyzed for evidence of suppressor cells. When MPBL were passed over G-10 Sephadex columns to remove monocytes, lymphocyte proliferative responses often returned to normal in lung cancer patients, suggesting an adherent suppressor cell. Unseparated MPBL or cells adherent to the G-10 columns were able to suppress normal individuals' responses to PHA, Con A or MLC. Some breast cancer patients had suppressor cells which could not be removed by the G-10 columns.

Rosette levels of lung cancer patients were taken monthly for six months after surgery to see whether the 29° rosette assay might be a useful prognostic tool. Among patients with 1/3 or more of their tests depressed during this period, a higher proportion relapsed than did those patients who had a lower incidence of depressed tests. These results suggested that repeated

rosette testing of lung cancer patients in the first several months after surgery might help to more accurately stage the patients.

Indirect migration inhibition tests of untreated lung cancer patients, of lung cancer patients receiving radiotherapy, and of normal donors were made with extracts of pleural effusion cells from a patient with adenocarcinoma of the lung and with tissue culture cells from a squamous cell carcinoma of the lung. Among the untreated patients, 60-70% produced leukocyte inhibitory factor (LIF) to these antigens. Most patients receiving radiotherapy, on the other hand, were not capable of producing LIF. Of considerable interest was the observation some normal donors who had close contact either with lung cancer patients or with tumor or blood specimens produced LIP, whereas normal donors without such contact were not reactive.

An improved radioimmunoassay (RIA) which clearly measures normal alpha-fetoprotein (AFP) was developed. This RIA has been used to measure serum AFP levels in patients with hepatocellular carcinoma. When patients with elevated levels of serum AFP responded to therapy, there was a concomitant drop in AFP. The drop in AFP level was generally a more sensitive and rapid indication of response than were the usual clinical parameters. In addition, AFP level usually gave the first evidence of resistance to chemotherapy or recurrent tumor growth.

A human lung tumor associated antigen (LTAA-2) was purified from the saline extract of an adenocarcinoma of the lung, and a double antibody RIA was set up.

The Laboratory of Cell Biology, directed by Dr. Lloyd Law, conducted research on the chemical and structural nature of antibodies, immunology, viral oncology and mammalian cellular genetics.

Studies of the primary and three-dimensional structure of the antibody molecule focused on analyzing several antigen binding myeloma proteins. These proteins have been shown to have antigen binding activity for a series of haptens including phosphorylcholine, galactan and levan. A molecular model was constructed of the antigen binding site for one of the phosphorylcholine binding proteins. The light chain sequence of a second such protein was completed as well. Problems in isolating the peptides for the heavy chains of the galactan binding proteins were solved, and three heavy chains from this group were nearly completed.

The heavy chains of papain-solubilized $H\text{-}2^a$ antigens were purified to homogeneity and chemically characterized. The molecules were found to have 290 amino acid residues, two disulfide briges and one free cysteine residue, and homologous NH_2- and COOH-terminal sequences. The NH_2-terminal sequences of the H-2 chains closely resembled those of HLA. However, the solubilized mouse and human histocompatibility antigens differed in chain length, COOH-terminal sequence and cysteine content. In addition, a serum component which appears to be shed H-2 antigen was purified from serum of Swiss BALB/c mice.

This molecule could represent an H-2 which is negative for alloantigenic specificities but still retains the basic structure of H-2.

Cross-reactivities of TSTA of SV40-induced neoplasms have been reported for several species, including man, rat, sheep, hamster and mouse. More recently, though, neoplasms induced in hamster by SV40, BK and JC viruses were found to have their own distinct tumor rejection antigens which do not cross-react. The T-antigens of these neoplasms were reported to be antigenically related, however. A new technique for obtaining TSTA from SV40-induced neoplasms of the mouse was developed which does not require solubilization with detergents. Homogenization of dissociated cells and gel filtration yields a water-soluble antigen which retains all the biologic and immunogenic characteristics of the intact cell: immunization is achieved easily and is specific for SV40-induced neoplasms, the immunity is stable and long-lasting (150 days) and there is no "overloading" with large doses of antigen.

The possibility that H-2 antigens in a modified form may be tumor specific antigens of the transplantation type was investigated. Because alien H-2 specificities had been described in several MCA-induced sarcomas in BABL/c mice, a series of seven such neoplasms was examined. In vivo immunogenicity assays yielded no evidence for alien H-2 determinants. In limited studies with serologic assays, no evidence for any H-2 determinants was encountered except those public and private specificities determined by H-2D^d and H-2K^d loci. It was concluded that all these sarcomas have individually distinct TSTA and that no detectable modified or derepressed H-2 antigens function as TSTA.

More has been learned about certain events which occur during the formation of intracisternal A particles (IAP). Six methylases were shown to be associated with IAP, and using E. coli phenylalanine tRNA as an acceptor RNA, these enzymes were shown to be indistinguishable from cellular enzymes. The specific activity of these enzymes increased about two-fold when IAP cores were prepared by stripping the outer envelopes with 1% Triton X-100, which suggests that the methylases are present inside the particles and not just absorbed to the surface. These cellular enzymes are probably packaged within IAP during the synthesis process.

In previous experiments, ecotropic virus isolated from SJL/J mice was not shown to have a direct role in the etiology of reticulum cell neoplasms (RCN). The possibility that a xenotropic virus is involved in this system was thus investigated. Studies in the AKR mouse indicated that an increased activity of ectopic virus, which by itself is not leukemic, may lead to activation of xenotropic virus and manifestation of leukemia, and to production of dual tropic recombinant viruses. The presence of xenotropic virus in the normal SJL/J mouse embryo and RCN suggests the existence of such an oncogenic mechanism, even though no dual tropic virus has thus far been isolated in this mouse.

In the area of mammalian cell genetics, it was shown that chloramphenicol resistance (CAP-r) could be transmitted to chloramphenicol sensitive (CAP-s) cells by treatment with purified mt-DNA alone. The mechanism of this "transformation" was studied to determine if a recombination-like event was involved, or if repopulation of the whole CAP-s cells mt-DNA by the entire CAP-r molecule occurred. In all four transformed cell strains examined, there was no evidence of persistent CAP-r mt-DNA still present in the treated cell lines. This implied that a recombination-like mechanism supersedes when CAP-r mt-DNA of one subspecies is used to "rescue" a CAP-s cell strain of another species. It was also established that all of the "transformants" are capable of transmitting the CAP-r phenotype by cytoplast fusion and they therefore represent changes in the cytoplasmic markers.

The research program of the Metabolism Branch, directed by Dr. Thomas Waldmann, has sought to define host factors which result in a high incidence of neoplasia, and to determine the physiological and biochemical effects that a tumor produces on the metabolism of the host.

A main area of concern has been the definition of cellular differentiation, interaction and biosynthesis involved in the specific circulating immune response. Studies have been aimed at defining the defects of regulatory T cell and macrophage interaction with B lymphocytes and plasma cells which occur in patients with primary immunodeficiency diseases associated with a high incidence of malignancy, and in patients with malignancies of the T or B lymphocyte system. A series of new activators of B cell immunoglobulin synthesis has been identified, including Nocardia opaca extract, staphylococcal-phage-lysate, staphylococcal organisms of the Cowan's strain A and streptolysin O. In addition, a new approach to looking at B cell differentiation has been developed which allows a precise count of the lymphoid cells secreting each class of immunoglobulin. This technique, called the reverse hemolytic plaque assay, has been employed in studies of normals and patients with a variety of disorders. Another project demonstrated that immunoglobulin secreting cells are a normal constituent of the lymphoid cells of man's lower bronchial alveolar tree. These cells may have an important role in local pulmonary immune defense and may contribute to the lung's sensitivity to allergic reactions.

Homogenous populations of T cells from patients with T cell leukemias have been studied to see whether they retain the capacity to help or suppress the synthesis of immunoblobulins from lymphocytes derived from normal individuals. A critical question is whether suppressor cell activity is generated by a single population of T cells or if the interaction of different populations of T cells is necessary. An unusual patient with acute lymphoblastic leukemia and hypogammaglobulinemia prior to therapy was helpful in addressing this question. Neoplastic T cells from this patient functioned as pro-suppressor cells. His leukemic T cells produced no detectable immunoglobulin and depressed the immunoglobulin production of co-cultured normal unseparated lymphocytes by 85-100% in the presence of pokeweed mitogen. However, suppression was only observed when cooperating normal T cells were present. Prior irradiation of either the leukemic cells or the cooperating normal cells nullified the suppressor effect. Therefore, it was shown that

an interaction between at least two different T cell subsets is required for generation of suppressor-effector T cells in man.

T cells have been found to encompass subpopulations of lymphocytes with different and at times opposing functions, and it has been suggested that there are two distinct population--Tμ and Tγ-- with different immunoregulatory potential. In the past year, studies have revealed that a rigid classification of T cells into so-called Tμ and Tγ cells is inappropriate. Rather, it was observed that normal T cells may express Fc-IgM and Fc-IgG receptors simultaneously under appropriate conditions, and there may be a transition of Tγ cells into Tμ cells. In addition, it was not possible to categorize Tγ cells as having an exclusive suppressor function and Tμ cells as having an exclusive helper function. These experiments pointed out that membrane Fc-IgG and Fc-IgM receptors should not be used indiscriminately to predict immunoregulatory function.

Gluten-sensitive enteropathy(GSE), a disease in which a disordered immune response is related to genes associated with the major histocompatibility complex as well as to genes on a separate chromosome, was further investigated. Previously it was observed that about 80% of GSE patients bear the HLA-B8 histocompatibility antigen. A similar incidence of this antigen was seen in dermatitis herpetiformis, a skin disease associated with gastrointestinal lesions essentially identical to those found in GSE. Studies of dermatitis herpetiformis patients revealed that there are two forms of the disease: one associated with granular IgA deposits of the skin, gastrointestinal abnormalities and elevated HLA-B8, and one involving linear IgA deposits in the skin, no GI abnormalities and a normal HLA-B8 incidence.

To better understand how the immune system recognizes and destroys foreign cells, early antigen non-specific cytotoxic responses--which precede specific immune recognition--were studied. Employing a model which measured pure monocyte-mediated killing, it was observed that development of cytotoxicity is due to an *in vitro* inactivation of a potent suppressor cell found in fresh cells. Several common plant mitogens, especially pokeweed mitogen, acted to dramatically stimulate cellular suppression of non-specific monocyte-mediated cytotoxicity. Patients with common variable hypogammaglobulinemia, ataxia-telangiectasia and the Wiskott Aldrich syndrome were noted to have defective non-specific monocyte killing. Evidence pointed to a distinct defect for each disease.

Studies of porphyrin metabolism have involved giving hematin to patients suffering acute attacks of porphyria. All patients responded to intravenous hematin with a decline of porphyrin precursor excretion and considerable improvements in abdominal pain, tachycardia and hypertension. No side effects of hematin were observed, other than occasional venous irritation.

The work of the Laboratory of Pathology and the Dermatology Branch is discussed in this section. The Diagnosis Program, a collaborative, extramural effort, will not be reported here. For information on the clinical research of the Metabolism Branch, please refer to the preceding section on immunology.

The Laboratory of Pathology, headed by Dr. Louis Thomas, conducted diagnosis and research activities in the areas of hematopathology and immunopathology, electron microscopy and histochemistry, viral oncology and cell biology, comparative pathology and carcinogenesis, automation of diagnostic cytology equipment, and clinicopathologic studies of a variety of diseases. The laboratory staff provided diagnostic services for Clinical Center patients as well.

The Surgical Pathology and Postmortem Service accessioned about 6,779 surgical specimens and biopsies and 224 autopsies in the past year. This work involved preparing more than 65,000 slides, 42,000 H & E stained sections, nearly 8,000 special stains and 531 frozen sections.

Studies of the human herpesviruses (HSV) and papovaviruses yielded information about the topology of the HSV induced Fc receptor, which appears to be mingled with other viral antigens. Viral-induced proteins were precipitated with antigen-antibody complexes. The binding of viral protein was found to be saturable and dependent on the presence of the Fc region of the antibody molecule.

The Cytopathology Section provided diagnostic services in exfoliative cytology, medical genetics and fine needle aspiration. Chromosomal analysis included standard karyotyping and special banding techniques for examination of individual chromosome pairs. About 3,800 cytology specimens were accessioned, representing over 9,000 smears which were examined and reported. About 1/4 were cervical and vaginal specimens. The remainder included ascitic, pleural, gastric, prostatic and spinal fluids, bronchial specimens, wound washings and fine needle aspirations.

The Quantitative Cytology Section focused on the use of cell sorters and flow microfluorometry (FMF) techniques. Data from FMF experiments have been analyzed with a computer-based decision tree logic program and also with Fourier transform analysis. In the decision tree program, 80.2% of 124 cytology specimens were analyzed correctly, with a false positive rate of 27% and a false negative rate of 9.6% The Fourier analysis yielded 79.6% correct diagnoses, 15% false positives and 30% false negatives. Work to refine both methods of cytologic screening is continuing.

In another project using FMF techniques, chicken erythrocytes were used as an internal biological standard for analysis of DNA content of normal and neoplastic cells. DNA fluorescence intensity changes in drug-perturbed L1210 ascites cells were studied. Drug-treated cells showed changes in

fluorescent intensity relative to the standard which were not seen in the controls.

A radioimmunoassay technique was created to determine accurately an Le^b-active oligosaccharide in urine. Using this method to examine the urine of 138 pregnant or lactating women with different Lewis blood types, it was shown that excretion of this oligosaccharide reaches a maximum in the third trimester and early postpartum period, and continues until lactation ends. Oligosaccharide may, the evidence suggested, originate in the mammary gland. The amount secreted was correlated to the Lewis blood type. Experiments were also begun to isolate the HY antigen thought to be involved in sexual differentiation of the gonads.

The Hematopathology Section continued its retrospective study of all non-Hodgkin's lymphoma patients treated at NIH, in order to characterize the population, study the disease's natural course and current classifications, and correlate treatment and staging data with survival.

Work has continued on epithelial-mesenchymal (stromal) interactions and their relationships to neoplasia. Polyoma virus-induced (PV) neoplastic changes in mouse salivary gland epithelium were found to vary widely depending on the presence or absence of stromal cells. The evidence suggested that stroma may inhibit tumor progression and influence the type and degree of differentiation expressed by neoplastic cells. For example, neoplasms from salivary gland epithelium transformed in vitro by PV in the absence of stroma were undifferentiated. Neoplasms induced by PV in the presence of stroma showed some differentiation into glandular, squamous or bone and cartilage forming components.

Investigations of the causes of lung cancer have centered on air pollutants, tobacco smoke condensates, asbestos and other fibrous particles. Although the PAH fraction in cigarette smoke was shown to be by far the most carcinogenic component, other components--particularly the acidic fraction and aqueous methanol subfraction--augmented carcinogenesis and could themselves induce pulmonary tumor. The PAH crude extract of air particulates also proved to be highly carcinogenic for the lung.

The durability and fiber size of asbestos and fibrous glass were previously shown to be critical to the production of pleural and pulmonary neoplasms in rats. In the past year, pleural neoplasms were also produced in mice exposed to asbestos and fibrous glass. Preliminary data suggested that particulates with diameters less than 0.02 μm or more than 3μm are not carcinogenic.

The relevance of polypeptide marker to the neurovirulence of poliovirus vaccine was investigated. Vaccine mutations rather than host factors alone may have been responsible for a case of vaccine-associated paralysis in an immunodeficient child.

In April 1978 the Image Processing Unit was transferred from the Quantitative Cytology Section to the Office of the Chief. Projects in the

area of nucleic acid biochemistry have capitalized on image processing in data acquisition, analysis and representation. Progress was made on measurements of single molecular images which are needed to produce secondary structure maps of nucleic acid molecules. Structure maps were generated from preprocessed images of non-looped, non-branched SV40 virus images.

In the past year the Pathology Technology Section processed over 28,000 pieces of animal tissue, prepared over 29,000 H and E slides and over 4,700 special stains.

The Dermatology Branch, headed by Dr. Marvin Lutzner, performed clinical and basic research into the etiology, diagnosis and treatment of diseases involving the skin and the host's responses to these diseases. In its role as dermatology consultant for the Clinical Center, the Branch saw about 1,000 patients last year.

The use of synthetic retinoids in treating skin cancer and diseases of keratinization was studied further. Patients with treatment-resistant keratinization disorders were treated with oral 13-cis retinoic acid, and those with congenital ichthyosiform erythroderma, lamellar ichthyosis, Darier's disease, pityriasis rubra pilaris and cystic acne had excellent responses. Patients with epidermolytic hyperkeratosis responded only partially, however, and those with psoriasis, nevus comedonicus, X-linked ichthyosis and Netherton's syndrome either did not respond or worsened. An oral synthetic aromatic derivative of retinoic acid was similarly tested and found to be more effective and less toxic than 13-cis retinoic acid.

The role of DNA repair processes in human diseases, carcinogenesis and aging was investigated using cells from patients with xeroderma pigmentosum (XP). All the patients had defective DNA repair, premature aging of the skin and cutaneous malignancies. The effectiveness of DNA repair was measured with assays of post-irridiation colony-forming ability (CFA). A striking correlation was observed between the level of effectiveness of DNA repair in XP and the severity of XP-associated neurological abnormalities. These results seemed to support the theory of "intrinsic mutagenesis" which hypothesizes that error-proneness in key DNA repair or replication enzymes is responsible for carcinogenesis and for a variety of degenerative diseases as well as for normal aging of human tissues. The study also confirmed recent reports that cells from patients with Cockayne's syndrome--which entails cachetic dwarfism, neurological abnormalities and photosensitivity--have decreased post-irridiation CFA.

A sensitive 10-day transfection assay for murine sarcoma and leukemia viruses was developed for use with integrated and unintegrated DNA. The efficiency of transfection depended on several criteria: size of infectious and carrier DNA, concentration of DNA, cell density, ph of precipitation medium and use of dimtheylsulfoxide. DNA was titrated almost as easily as intact virus using this technique.

Pemphigus, herpes gestationis, bullous pemphigoid and dermatitis herpetiformis were the means of studying immunopathologic mechanisms involved in inflammatory skin diseases. Serum and *in vivo* bound antibodies were identified and characterized in these diseases. Gastrointestinal abnormalities similar to those seen in gluten sensitive enteropathy were investigated in patients with dermatitis herpetiformis. Gluten withdrawal from the diet was shown to reverse the skin disease in many patients.

Another area of interest was the production of abnormal proteins in malignant melanoma. The melanosome in normal mouse tissue was found to contain multiple proteins, many of which are loosely bound and easily extracted. Melanosomal proteins from melanoma tissues differed structurally from those of normal tissue; several proteins seemed to be completely missing. More importantly, it was noted that many of the proteins in melanoma melanosomes are unique and are not found in normal melanin granules. A slight difference of 10,000 molecular weight was also observed between the normal and abnormal proteins.

I. INTRODUCTION

II. BIOCHEMISTRY OF GENE EXPRESSION SECTION (Dr. E. B. Thompson, Head)

- A. Studies on the Cell Biology of Steroid Action
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III. BIOSYNTHESIS SECTION (Dr. E. L. Kuff, Head)

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Annual Report of the LABORATORY OF BIOCHEMISTRY, National Cancer Institute
October 1, 1977 through September 30, 1978

I. INTRODUCTION

This was a year of innovation for the Laboratory of Biochemistry. It was the first year in which we had our own P3 facility available for studies on molecular cloning--a facility that has been in almost constant use by a number of our research groups. It was the year in which we finally saw the installation of our new electron microscope and "smart" computer terminal. And, most important, it was the year when Dr. Igor Dawid and his research group from the Carnegie Institution moved into the space that had been renovated for them. It is also a pleasure to report that interactions among the scientists of our various Sections, as well as among scientists of this Laboratory and other Laboratories, have flourished. Enthusiasm runs high.

The Laboratory of Biochemistry is divided into six Sections. The Biochemistry of Gene Expression Section studies control of gene expression in eukaryotic cells and its application to clinical medicine. The effects of steroid hormones on enzyme induction and cell function in tissue culture are investigated, along with steroid receptors in cells from patients with leukemia and breast cancer. The Biosynthesis Section is concerned with the relationship between cell function and organization at the molecular level. Current studies deal with enzymatic aspects of DNA and collagen biosynthesis and with the regulation and functional correlates of endogenous retrovirus expression in normal and transformed cells. The Cellular Regulation Section studies regulation of gene expression in eukaryotes at the molecular level. The central problem under investigation is the mechanism by which estrogen induces the synthesis of egg yolk phosphoprotein in rooster liver, with special emphasis on the molecules involved in the interactions of hormone, hormone receptor, RNA polymerase and chromatin that result in transcription of the phosphoprotein gene. The Developmental Biochemistry Section is involved in studies on the structure and transcription of the eukaryotic genome. Studies are conducted on frog mitochondrial DNA, Drosophila ribosomal DNA, the structure of the gene coding for frog yolk proteins, and the pattern of gene expression in embryos. The Nucleic Acid Enzymology Section is concerned with the structure and metabolism of nucleic acids and nucleotides. Systems under investigation include the structure and origin of host-substituted defective simian virus 40, the organization of the monkey genome and the control of cyclic AMP level and function by Ca^{++}-dependent regulatory protein. The Protein Chemistry Section emphasizes the physical, chemical, and biochemical aspects of proteins and subcellular particulates involved in the synthetic, control, and differentiation systems of organisms. Problems currently under study include transfer of isolated chromosomes to mammalian cells, granulocyte development, fractionation of cells involved in cellular immune reactions, and techniques for fractionation of proteins.

II. BIOCHEMISTRY OF GENE EXPRESSION SECTION (Dr. E. B. Thompson, Head)

Actions of steroid hormones and molecular studies of transcription have been the foci of work in 1977-78. Studies of steroid action can be considered in three categories: cell biology, biochemistry, and clinical.

A. Studies on the Cell Biology of Steroid Action.

1) The CEM line of human acute lymphoblastic leukemic (ALL) cells has been employed further to examine the nature of lymphocytolysis of ALL cells by glucocorticoids. By fluctuation analysis, these cells spontaneously develop steroid resistance at ~ 1 event per 10^5 cells per generation. In all 53 resistant clones examined so far, reduced numbers of steroid receptors, with defective nuclear transfer have been found.

Cell cycle studies have shown that steroids produce irreversible arrest and death in G_1.

The interactions of drugs commonly employed in the therapy of ALL have been studied. Prednisolone plus vincristine or methotrexate were slightly synergistic, whereas prednisolone plus 6-mercaptopurine were antagonistic.

2) Several clones of spontaneous HTC variants, containing tyrosine aminotransferase (TAT) not inducible by glucocorticoids, have been tested for their responses with respect to five other functions known to be affected by such steroids in wild type cells. They were found to be resistant also to TAT induction by steroid plus dibutyryl cyclic adenosine monophosphate and to suppression of cAMP phosphodiesterase. Induction of glutamine synthetase, suppression of plasminogen activator and inhibition of α-amino-isobutyric acid uptake all occurred as in wild type cells.

3) Lines of mouse lymphoma cells bearing discrete markers of T cell differentiation (Ly antigens) were characterized with respect to their glucocorticoid receptors, and their growth was shown to be inhibited by steroids.

4) Clones of steroid-resistant L cells with apparently normal glucocorticoid receptors were obtained and characterized.

B. Studies on the Biochemistry of Steroid Action

1) The pre-activated and activated forms of the glucocorticoid-receptor complex, shown to be separable by DEAE-cellulose chromatography, were more fully characterized.

2) Affinity labels for glucocorticoid receptors are being developed, and several compounds appear promising.

3) Reagents and methods to study the control of prolactin production by steroids (and other hormones) are being developed. Using a line of cells derived from cell hybrids between L cells and pituitary adenoma cells,

we have isolated several clones of high prolactin producers. Translation of mRNA from these clones in a wheat germ cell-free system yields immunoprecipitable prolactin. We have prepared cDNA from these mRNA extracts and are engaged in further characterizations of the cDNA.

C. Studies on Steroid Receptors in Leukemia and Breast Cancer

1) In the abnormal suppressor T cells of Sezary's syndrome, a rare form of erythrodermic leukemia, glucocorticoid receptors have been identified and characterized. Induction of glutamine synthetase has been shown to be a marker for functional receptors in these cells. This work and that in the Ly-marked mouse lymphomas mentioned above, is the first which defines the steroid receptors in, and responses of, functional subsets of T lymphoblasts.

2) Data on estrogen, androgen, glucocorticoid, and progestin receptors has been analyzed in a large series of breast cancer patients. It was found that lack of estrogen receptors correlated with a high response rate to chemotherapy, while presence of the receptors correlated with a poor response rate. The raw data are being analyzed currently for other correlations.

D. Molecular Studies of Transcription

1) The mRNA populations in differentiating chick muscle cells in culture were analyzed by nucleic acid annealing, and specific classes of mRNA's associated with differentiation were defined.

2) A method for identifying interactions of specific mRNAs and DNAs in translation systems was developed. Termed "hybrid arrest of translation," the method has been used, along with DNA sequencing of known restriction fragments, to study the gene arrangement and transcription pattern of various mRNAs.

3) The effects of mRNA modification on translational efficiency have been studied using defined prokaryotic mRNAs. These studies included examination of 7-methyl G capping of mRNA and of the eukaryotic vs prokaryotic ribosomal binding sites on prokaryotic mRNA.

III. BIOSYNTHESIS SECTION (Dr. E. L. Kuff, Head)

A. Biosynthesis and Secretion of Collagen. Dr. Peterkofsky's unit has continued studies on a reducing cofactor for prolyl hydroxylase which can be measured in extracts prepared from transformed cells. The cofactor has been localized in the microsome-containing particulate fraction of cell homogenates. Prolyl hydroxylase is known to be contained in microsomes; and association of the cofactor with this subcellular fraction is consistent with a functional relationship between it and the enzyme. Studies on the localization of lysyl hydroxylase have also continued. In contrast to prolyl hydroxylase, which is located almost entirely within the microsomal cisternae, only 30% of the lysyl hydroxylase is intracisternal; the bulk of this activity is firmly associated with the microsomal membrane through ionic bonds. Finally, some newly initiated studies on collagen secretion have shown that this process

can be strongly inhibited by treating the cells with local anesthetics. Inhibition was not due to interference with collagen synthesis, prolyl hydroxylation or depolymerization of microtubules, although studies by others have implicated local anesthetics in disruption of microtubule function.

B. DNA Synthesis in Mammalian Cells. Dr. Wilson has continued his study of enzymatic mechanisms in DNA synthesis. He and his coworkers have purified to near-homogeneity several mouse proteins important for in vitro analysis of DNA replication. These include the α- and β-DNA polymerases and DNA unwinding protein. The α-polymerase of mouse myeloma cells has a native molecular weight of about 300,000 and contains at least 4 subunits; the β-polymerase is a single 40,000 dalton polypeptide chain. Steady state kinetic studies of the β-polymerase have provided data pointing to a sequential mechanism for DNA formation. Magnesium activation of this reaction does not change the K_m for either dNTP or template-primer, suggesting that Mg^{2+} and the substrates bind independently to the enzyme and that Mg^{2+} does not strongly affect binding of the substrates. The DNA unwinding protein is a polypeptide of about 25,000 molecular weight; it exhibits micro-heterogeneity on isoelectric focussing or high resolution SDS-polyacrylamide gel electrophoresis. This protein stimulates the in vitro replication of poly(dA) by α-polymerase 2- to 3-fold, and depresses the T_m of poly[d(A-T)]. Investigation of the in vitro activity of purified DNA replication proteins is being initiated using polyoma replicative intermediates as well as a single-stranded closed circular bacteriophage DNA. In collaborative studies with Dr. B. Carter (NIAMDD), Dr. Wilson has obtained evidence for a striking induction (60-fold) of α-polymerase activity in contact-inhibited monkey kidney cells on infection with adenovirus 2.

C. Cellular Interactions in the Immune Response. In her final year of service with the National Cancer Institute, Dr. Shelton has actively pursued her studies on the effects of metabolic inhibitors and temperature changes on the surface topography and internal structure of mouse lymphocytes, macrophages and mast cells. In the case of peritoneal lymphocytes and macrophages, she has shown that the structure of the cytoplasmic cortex and the microvilli is supported by a filamentous network consisting principally of actin. This actin meshwork is greatly reduced when cells are treated with diamide, a compound that eliminates microvilli, and is accentuated when cells are treated with azide, a compound that increases microvillus length in these cells. On omental mesothelial cells, the density of microvilli can be increased or decreased by raising or lowering the ambient protein concentration. Certain types of protein are more efficient effectors than others. Concomitant with the microvillus response is the appearance of 150-400 nM diameter pits in the cell surface; these are found most frequently at the base of microvilli. In other studies, uncouplers of oxidative phosphorylation (NaN_3, oligomycin and dinitrophenol) were found to cause reversible polymerization of microtubules in the cytoplasm and nuclei of mast cells but not of macrophages and lymphocytes.

D. Structure of Macromolecules. In collaboration with scientists elsewhere, Dr. Shelton had characterized the enzyme pyruvate oxidase by transmission electron microscopy of negatively stained preparations. The molecule

consists of 4 tightly packed subunits and resembles a sphere flattened at both ends. It is 11.5 nm high and 12.4 nm in diameter. Dr. Shelton has also examined one of the regulators of the complement "cascade", C3c-C4b inactivator co-factor. This 10^6 dalton protein closely resembles IgM in its structure.

E. Intracisternal Type-A Particles. Drs. Lueders and Kuff extended their study of these intracellular virus-like structures found in many types of mouse tumor cells. The polyadenylated RNA species associated with neuroblastoma and myeloma A-particles have been characterized by electrophoresis in agarose gels under fully denaturing conditions (10 mM methyl mercuric hydroxide). A 35S species approximately 8500 nucleotides in size, is uniformly present; this appears to be a genomic RNA since it can direct the in vitro synthesis of the major A-particle structural protein (experiments with Drs. B. Paterson and S. Segal). Other discrete smaller RNA components (24 to 32S) are present in varying proportions in A-particles from different sources; in the case of one myeloma, the predominant 28S RNA was also found to be active in coding for the A-particle structural protein. Representative cDNAs were prepared from isolated 35S and 32S A-particle RNAs of neuroblastoma and the myeloma 28S RNA species. The 35S RNA contained all of the sequences detected in the smaller molecules. Sequences homologous to the 35S RNA were reiterated more than 500 times per haploid genome of the laboratory mouse, Mus musculus; related but divergent sequences were found to the extent of 25 copies or less per haploid genome in the DNA of Asian species Mus cervicolor and Mus caroli. No homology was found between A-particle RNA and the high molecular weight RNA of mammary tumor virus or a wide variety of ecotropic and xenotropic type-C viruses of M. musculus. On the other hand, the A-particle RNA showed a partial sequence homology (about 30%) with a novel type of extracellular retrovirus first isolated from cells of M. cervicolor by Callahan et al. in 1976. Evidence suggests that A-particle-related genetic information is widespread in the genus Mus and may have contributed to the formation of a replication-competent class of retroviruses in an evolutionary ancestor of the Asian species.

IV. CELLULAR REGULATION SECTION (Dr. R. F. Goldberger, Head)

A. DNA Sequence Organization in the Chicken and Avian Evolution (Francine Eden). Our present knowledge of DNA sequence organization in the chicken indicates that this genome represents a pattern intermediate between the extremes of DNA sequences organization known in other eukaryotes. About one half of the chicken genome consists of a regularly alternating pattern of single copy and repetitive sequences, with the average length of single copy sequences about 4500 nucleotides. The other half of the genome seems to consist almost entirely of long single copy regions uninterrupted by repeated sequence elements for very long distances. Thus, the chicken genome can and has been preparatively fractionated into interspersed and non-interspersed portions. In addition, we have determined that the chicken genome contains an unusually small fraction of repeated DNA, and that these repeated sequences are much longer than the short (300 nucleotide) repeated sequences typical of most eukaryotic genomes. We have applied a new combination of preparative steps aimed at isolating fractions of the chicken genome that have been evolutionarily conserved. Using this procedure, we obtained a fraction of

chicken repetitive DNA that represents sequences conserved during at least 60 million years of avian evolution. By re-hybridization of this conserved fraction with chicken DNA and the DNA of other avian species, we were able to determine that although the sequences themselves have been conserved, their repetition frequencies in the respective genomes have undergone a hundred-fold change during the course of evolution. This study provides a new clue about the function of repetitive DNA: the nucleotide sequences themselves are probably more important than the number of copies of any particular sequence, since it is this feature that has been conserved evolutionarily.

B. Transcription of Yeast Ribosomal DNA in E. coli (Richard Kramer, Joan Smallwood). Bacteriophage λ carrying the region of DNA coding for the ribosomal RNAs of yeast has been employed in a study of the expression of yeast genes in bacteria at the level of RNA synthesis. Hybridization assays show that yeast rDNA is transcribed in Escherichia coli, even in the absence of phage RNA synthesis. In addition, the transcription occurs on only one strand of the DNA, the same strand from which yeast rRNA is transcribed. Thus, the transcription of yeast rDNA in E. coli is initiated in the yeast DNA and shows some specificity. However, attempts to detect unique yeast transcripts in E. coli by preparative hybridization to cloned yeast rDNA followed by gel electrophoresis have been unsuccessful. Electron microscopic mapping of these transcripts does show RNA of significant size homologous to the yeast rDNA, suggesting that the reason why species of unique sizes (necessary for detection on gels) are not present is that there is some non-specific transcription initiation or termination or some partial degradation of the transcripts. Quantitative analysis of the electron microscope data is underway to determine which possibility is most likely.

C. The Estrogen-Responsive Domain of the Rooster Liver (Roger Deeley, Richter King, and David Udell). Homologous, RNA excess, cDNA complexity analyses have indicated that approximately 14,000 different species of mRNA are present in rooster liver both before and after treatment with 17β-estradiol. It has been determined from heterologous complexity analyses, between liver RNA from an estrogen-treated rooster and cDNA obtained from the liver RNA of a normal rooster, that essentially all those mRNA species present before treatment with the hormone can be found following hormonal stimulation, and that they remain in approximately the same proportions. Similar experiments using liver RNA from normal roosters and fractionated cDNA prepared from liver RNA obtained from estrogen-treated birds have demonstrated that treatment with estrogen appears to result in the extensive accumulation of a few mRNA species that can be found in the "abundant" and "intermediate" classes of mRNA. No significant changes have been found in the RNA population of the "complex" class which contains 95% of the different mRNA species of the liver. A cDNA probe has been prepared that is specific for the most abundant of the estrogen-inducible sequences. By analyzing the kinetics with which this cDNA probe hybridizes to its template RNA it has been possible to estimate that it is derived from a group of mRNA species that has a total complexity of approximately 15,000 nucleotides.

D. Purification of Chicken Albumin mRNA and Cloning of Albumin dscDNA (Jeffrey Gordon, Roger Deeley, Alan Burns, and James Christmann). Albumin mRNA has been shown by cell-free translation to represent approximately 15% by weight of the mRNA of rooster liver. During acute treatment with 17β-estradiol the relative concentration of albumin mRNA declines until it comprises approximately 8% of the liver mRNA population. This decline in relative concentration does not reflect a true decrease in the albumin mRNA content of the liver but rather is a result of dilution by other abundant mRNA species, such as vitellogenin mRNA, the synthesis of which is markedly dependent upon induction by estrogen. These results suggest that the regulation of albumin mRNA synthesis and degradation is not appreciably affected by the administration of estrogenic steroid.

Albumin mRNA has been purified approximately 33-fold from total liver RNA. Double-stranded DNA has been synthesized from such an RNA preparation and cloned in the EK2 host/vector system X1776/PBR322. Plasmid DNA isolated from this clone has been used to identify albumin mRNA present in preparations of total liver RNA following separation by electrophoresis on methylmercury-agarose gels. It has been demonstrated that albumin mRNA is approximately 2600 nucleotides long and that it specifies a form of preproalbumin that has a molecular weight of 72,000. The RNA has been shown to be approximately 600 nucleotides longer than the minimum length necessary to specify preproalbumin.

E. Kinetics of Vitellogenin mRNA Following Estrogen Stimulation and Cloning of Vitellogenin dscDNA and Genomic DNA (Roger Deeley, Jeffrey Gordon, Alan Burns, and Robert Goldberger, in colaboration with Shirley Tilghman and Philip Leder, LMG, NICHD). It has been determined from cDNA hybridization studies that the livers of normal roosters contain from 0-5 molecules of vitellogenin mRNA per cell. An increase in vitellogenin mRNA concentration was detectable within 30 minutes of administering either a primary or secondary injection of 17β-estradiol. The rate of accumulation of mRNA following a primary injection, however, was found to be biphasic. Vitellogenin mRNA accumulated at a rate of only 50 nucleotides $sec^{-1}.cell^{-1}$ for the first 4 hours following injection of the hormone and then the rate of accumulation increased during the next 8 hours to reach 350 nucleotides $sec^{-1}.cell^{-1}$. In contrast, a secondary injection of hormone administered to a completely withdrawn bird was found to elicit an initial rate of accumulation of 350 nucleotides $sec^{-1}.cell^{-1}$ which subsequently increased to approximately 540 nucleotides $sec^{-1}.cell^{-1}$. Following both a primary and secondary injection of hormone vitellogenin mRNA decayed with an apparent half-life of 30 hours. The increased vitellogenic response to secondary stimulation was found to be proportional to the increase in vitellogenin mRNA concentration. The results indicate that primary treatment with estrogen results in a long lived alteration either in the availability of the vitellogenin gene for transcription or in the persistence of a mechanism for stabilizing vitellogenin mRNA during the first few hours of the vitellogenic response.

Chicken DNA was digested with the restriction enzyme EcoRI. The aggregate size of the genomic DNA fragments that contain parts of the vitellogenin gene was found to be approximately 25,000 base pairs, indicating that the gene

is most probably interrupted by sequences not represented in the mature mRNA. Vitellogenin dscDNA sequences have been cloned in the host/vector system χ1776/PBR322. Approximately 125 vitellogenin clones have been obtained that contain fragments of vitellogenin DNA ranging in size from 100 to 1000 nucleotides. Plasmid DNA from several of these clones has been prepared and attached either to nitrocellulose or diazotized paper filters for use as probes in in vitro transcription studies.

F. Isolation and Characterization of DNA-Dependent RNA Polymerase II from Rooster Liver and Studies on Transcription by This Enzyme (Kathleen Mullinix, William Kastern, Juanita Eldridge, in collaboration with Henry Miller and Philip Leder, LMG, NICHD). DNA-dependent RNA polymerase II has been isolated by a new procedure. It is completely DNA dependent and transcribes both native and single-stranded calf thymus DNA. Experiments on the binding of radioactive α-amanitin have shown that in rooster liver there are two α-amanitin-binding proteins, having molecular weights of 640,000 and 580,000 and being present in a ratio of 2 (640,000): 1 (580,000) at all stages of enzyme purification. The RNA polymerase was used to transcribe the chimeric pBR322-β globin 2 plasmid. The entire plasmid, both pBR322 and the β-globin 2 sequences, were transcribed and there was no evidence of specific transcription of the β-globin 2 sequence. We have just begun to study the extent of specific transcription of SV40 by this enzyme. We have utilized restriction endonuclease digestion of SV40 DNA by BamHI and HpaII to generate fragments of DNA corresponding to early and late mRNA-coding regions. This digested DNA has been subjected to electrophoresis in agarose gels under denaturing conditions to separate strands. This material has been transferred to nitrocellulose filters by the Southern blotting technique and we can ask if the enzyme transcribes symmetrically or asymmetrically and if the enzyme starts at the in vivo origin of transcription.

G. Susceptibility of the Vitellogenin Gene to DNase I Digestion (Kathleen Mullinix, Marilyn Meyers, Roger Deeley, and Robert Goldberger). These experiments were designed to measure the susceptibility of the chicken vitellogenin gene to DNAse I digestion before, during, and after stimulation by estrogen. It is known that the vitellogenin gene is not transcribed in liver and that vitellogenin mRNA synthesis can be induced by estrogen. After withdrawal from estrogen, vitellogenin mRNA synthesis ceases.

To ascertain that the methods employed were working as expected, we first investigated the susceptibility to DNAse I digestion of the globin gene in chicken erythrocytes. We found that after digestion with DNAse I there was a depletion of the globin sequences, indicating that the globin gene had been digested from erythrocyte nuclei to an extent that made it unable to hybridize with globin cDNA. These results are in accord with the results of Weintraub and Groudine.

To test the susceptibility of the liver vitellogenin gene to DNAse I digestion, we tested control and DNAse I-digested nuclei from the livers of estrogen-stimulated roosters by hybridizing the nuclear DNA to vitellogenin cDNA. We found that there were no detectable differences in the extent of hybridization to vitellogenin cDNA between control nuclei and DNAse I-digested

nuclei, indicating that by this method we could not observe selective digestion of the vitellogenin gene by DNAse I under conditions in which we know that vitellogenin mRNA was being synthesized.

H. Development of an In Vitro Transcription System (Kathleen Mullinix, James Christmann, Juanita Eldridge, Marilyn Meyers, and Robert Goldberger). We have developed a procedure for isolating rooster liver nuclei that are active in transcription. This procedure involves the homogenization of liver in isotonic sucrose and purification of the nuclei. We have also begun to study transcription in liver chromatin from normal and estrogen-stimulated roosters. Nuclei are isolated and chromatin is prepared by lysis of nuclei in hypotonic medium. The chromatin is not sheared prior to its use as a transcriptional complex and the chromatin-directed RNA synthesis that we have studied has utilized only the endogenous, chromatin-associated DNA-dependent RNA polymerase.

Nuclei isolated by our procedures from rooster liver are pure as measured by phase contrast microscopy and are very active in transcription. We have found that nuclei from estrogen-treated rooster liver are approximately twice as active in transcription as are those from livers of untreated roosters. We have had considerable difficulty in isolating high molecular weight RNA from our preparations, since the levels of ribonuclease in these nuclei are so high. Chromatin isolated from rooster liver nuclei by our procedures is also active in transcription, utilizing the endogenous chromatin-associated RNA polymerases. An extensive series of experiments in which we used Hg·UTP and Hg·CTP to label newly synthesized RNA has convinced us that this method is not suitable for obtaining an accurate estimate of the amount of newly synthesized vitellogenin mRNA in nuclei. We found that even under rigorously denaturing conditions it was not possible to remove all of the contaminating endogenous vitellogenin sequences from the newly synthesized RNA. We have decided that the experiment to determine the amount of vitellogenin RNA sequences transcribed in vitro could only be done utilizing a cloned vitellogenin sequence and hybridizing ^{3}H-UTP or ^{32}P-UTP-labeled RNA synthesized in vitro to vitellogenin DNA sequence under conditions of vast DNA excess. Such a cloned vitellogenin DNA sequence has recently become available (See NCI ZO1 CB 5238-09 LB). As a control, we will test the ability of nuclei from normal and from estrogen-stimulated roosters to synthesize albumin RNA since albumin RNA synthesis is not be affected by estrogen. A plasmid containing the albumin DNA sequence has recently been prepared in this laboratory (See NCI ZO1 CB 5250-01 LB).

V. NUCLEIC ACID ENZYMOLOGY SECTION (Dr. M. F. Singer, Head)

A. Calcium Dependent Regulator Protein of Cyclic AMP Related Processes. The last year has seen the emergence of important ideas concerning the central role of the small protein called "calcium-dependent regulator" (CDR) in the control of various calcium-regulated physiological processes in animal cells. Claude Klee and her colleagues Paul Richman and Tom Crouch have been studying the properties of CDR itself as well as its role in two physiologically important systems -- namely, the regulation of cellular cyclic AMP levels and (in collaboration with R. Adelstein) the phosphorylation of myosin light

chains. Chemically modified but still active forms of CDR were prepared, characterized, and used to study the effect of binding of calcium ions on the conformation of CDR as well as on its interaction with proteins known to be involved in regulation of cyclic AMP levels. Two such proteins, cyclic nucleotide phosphodiesterase and cyclic nucleotide phosphodiesterase inhibitory protein were studied. CDR binds to both these proteins in a calcium-dependent fashion. Activation of the phosphodiesterase by CDR requires that at least three of the four possible calcium-binding sites on the CDR be occupied by the ion. Further, it was shown that the CDR binds to the catalytic subunit of the phosphodiesterase (molecular weight, 59,000). The other two subunits of the phosphodiesterase have molecular weights of 61,000 and 15,000, respectively, and were shown to be components of the inhibitory protein. Thus, a complex system of activator, inhibitory protein and enzyme, sensitive to calcium ion in the physiological concentration range and thus capable of very fine regulation is established. Myosin light chain kinases, from both smooth muscle and non-muscle cells were purified by affinity chromatography on CDR coupled to Sepharose, thus indicating the ability of CDR to bind to these two enzymes. Both these proteins are also activated by CDR.

B. Organization of Repetitive DNA Sequences in the African Green Monkey. Another group in the Section (Maxine Singer, Dinah Singer, Thomas McCutchan and Ronald Thayer, in collaboration with Martin Rosenberg of LMB, NCI) have continued work on the highly repetitive sequences in the genome of the African green monkey. Sequences of this class, collectively referred to as α-component, make up between 10 and 20 percent of the total genome. They are a set of sequences each 172 base pairs long and very similar in nucleotide sequence but the various members of the set differ from one another in one or a few base pairs. The number of members of the set and their relative abundance is not known, but overall the 172 base pair segment is repeated several million times in the monkey genome. A nucleotide sequence representing the most abundant residue at each of the 172 positions in all the members of the set taken as a whole has been determined. Preliminary data indicate that members of the set differ from one another in a nonrandom manner. These highly repeated sequences were shown to be organized into nucleosomes within chromatin. Further, it was demonstrated that the sequence itself does not instruct a unique positioning of the nucleosomal structure. The literature contains a great number of speculations on the function of such highly repeated sequences, but we remain unimpressed with all of them and there are no significant data supporting any of the current proposals. Characterization of this sequence has established the existence of two different classes of highly repeated sequences in eukaryote genomes: the complex class, of which α-component is one, where the repeat length is quite long; and the simple class, where the repeat length may be fewer than 10 nucleotides. The classical "satellite" DNAs are largely members of the "simple" sequence class.

C. Defective Variants of Simian Virus 40. One of the reasons for the interest in α-component sequences is that they frequently occur in defective variants of simian virus 40 that are recombinants between viral and host (cellular) DNA sequences. Almost the entire nucleotide sequences of two such defective variants of SV40 have been determined (Maxine Singer, Thomas McCutchan, Tateaki Wakamiya in collaboration with Martin Rosenberg of LMB,

NCI). Each of the variants contains a different member of the set of sequences comprising the α-component. Each of the variants contains less than a full 172 base pair α-component repeat unit and in each variant the α-component sequences overlap but start and stop at different positions within the 172 base pair repeat. Each variant also contains sequences tentatively identified as unique, or low reiteration frequency, monkey sequences: the sequences in the two variants are different. The sequences around the joints between monkey and SV40 segments do not indicate any striking homology or similarity. Thus, nonhomologous recombination mechanisms of an unknown nature were involved in the formation of the defectives.

VI. PROTEIN CHEMISTRY SECTION (Dr. E. A. Peterson, Head)

A. Chromosome Transfer. Dr. McBride and his colleagues have shown that after the transfer of functional chromosomal donor genes to recipient mammalian cells following the uptake of isolated metaphase chromosome in vitro, the transgenome can exist in at least two states in the recipient cells. The first is an unstable state in which there is a high frequency of loss of the transferred genes (10^{-1} per cell per generation), accompanied by a much lower frequency of conversion to a second state (10^{-5} per cell per generation) in which these genes are stable. The serial transfer of functional chromosomal donor genes by this process has provided evidence that stabilization of the transgenome involves its integration into the DNA of the recipient cells. Analysis of the segregation of a stably transferred genetic marker, HPRT, with chromosomes of recipient cells has indicated that integration of the transgenome can occur at multiple sites and does not require crossing over at the homeologous locus.

Efforts to determine the size of the transgenome have continued. Since the amount of donor DNA in gene transfer clones is very small, a two-step approach is being used. The first step involves the isolation and labeling of human X-chromosomal unique-sequence DNA from a human-mouse hybrid cell line in which that chromosome is the only human chromosome. This DNA will then be used as a probe to determine the amount of human X-chromosome present in various gene-transfer clones. The necessary preliminary studies and preparations have been completed and the labeling of the isolated DNA is under way.

B. Cellular Immune Reactions. Dr. Mage has prepared antibody (anti-CTL) specific for mouse cytotoxic T lymphocytes and their precursors and has used it to study the contribution of this T cell lineage to the graft vs host reaction (GVH) in vivo. Treatment of spleen cells with the antibody reduced the total GVH activity by about 30%. The majority of the affected cells appeared to be those that do not adhere to allogeneic target cell monolayers. Treatment of either thymus or spleen cells with the antibody had no effect on the synergy exhibited in the production of GVH splenomegaly by mixtures of these cells.

When thymus cells (unlike spleen cells) were treated with the antibody, some of the survivors generated CTL activity on culture in vitro, indicating

that the thymus may contain CTL precursors that have not differentiated sufficiently to be susceptible to the anti-CTL.

C. Granulocyte Development. Pursuing their studies of the inflammatory response as a model for the regulation of granulocyte maturation, Dr. Evans and his co-workers have found that normal guinea pig serum contains a heat-sensitive, nondialyzable factor that inhibits glycoprotein synthesis in bone marrow granulocytes in vitro, as shown by its effect on the incorporation of ^{14}C-glucosamine into the glycoproteins of these cells. Relative to normal serum, inflammatory serum has a marked stimulatory effect, reaching a maximum in serum withdrawn 8 hours after the initiation of inflammation in vivo. Available evidence suggests that the stimulation produced by inflammatory serum is due to the appearance in serum, during inflammation, of a factor that blocks the effect of the inhibitor in normal serum, rather than the result of a disappearance of the inhibitor from the serum.

Using gel electrophoresis in the presence of sodium dodecyl sulfate, Dr. Evans has found at least eight glycoproteins, ranging in molecular weight from 16,000 to 110,000, to be associated with leukocyte granules. All of these except one can be extracted from the granules with cetyltrimethylammonium bromide. Most of the broad band of periodate/Schiff-positive material that migrates at the dye front in such gel patterns has been identified as ceramide-like glycolipids that are removable by extraction with a mixture of chloroform and methanol.

D. Purification of Granulocyte Marker Proteins. Drs. Peterson and Torres have undertaken the purification of some of the proteins of guinea pig leukocyte granules for use as markers in collaborative studies with Dr. Evans on the regulation of the maturation of neutrophils. Lactoferrin, a glycoprotein specific to neutrophils within the bone marrow population but found in milk in much larger quantities, was isolated from guinea pig milk by chromatography and used to prepare a monospecific antiserum for radioimmunoassays. Because of uncertainty as to the suitability of lactoferrin as a marker for neutrophil granule synthesis, the purification of other granule proteins was pursued. Initial efforts employing CM-cellulose to fractionate granule proteins that had been extracted with sodium acetate at pH 4 were defeated by a tendency of lactoferrin to bind other granule proteins at relatively low salt concentrations. This was overcome by the use of copper-chelate chromatography, which permitted operation at a salt concentration high enough to prevent such association. Lactoferrin was thus separated from the other granule proteins and two of the latter were purified to apparent homogeneity. Unfortunately, these were not specific for neutrophil granules so purification of the other major proteins in the remaining fractions is continuing.

The residue left by extraction with acetate and citrate contains additional proteins of interest as potential markers. Several special detergents have proved to be effective in extracting these proteins, but true molecular dispersion under conditions permitting fractionation has not yet been achieved.

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COOPERATING UNITS (if any)

M. L. Lippman, J. Allegra, J. Mason, C/M;
A. Tashjian, Harvard Medical School; P. Dannies, Yale Medical School;
A. Kandutsch and H. L. Chen, The Jackson Laboratory; J. Kim, I/LMI;

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Biochemistry of Gene Expression Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
12	9	3

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A human leukemic cell line has been used to study steroid actions. Glucocorticoids stop these cells in the G_1 phase of the cell cycle, after which they die. Steroid resistant subclones have been isolated and are being analyzed. HTC cell variants have been found to lack coordinate control over 5 steroid-sensitive functions. Lack of estrogen receptors in human breast cancer has been found to correlate with high response rate to chemotherapy. Sezary syndrome cells have been found to contain glucocorticoid receptors, as have lines of mouse lymphomas bearing specific sets of Ly antigens. The pre-activated and activated forms of glucocorticoid receptors have been separated and factors influencing activation studied. Affinity labels for glucocorticoid receptors are being developed. Chromosome mapping of certain steroid-related functions has begun. Studies of prolactin mRNA have been developed.

PHS-6040
(Rev. 10-76)

Cooperating Units (Cont'd)

D. K. Granner, U. Iowa Medical School; T. D. Geleherter, U. Michigan Medical School; D. Salomen, D/LDBA; S. Simons, A/LC; B. J. Fowlkes, C/LP; A. Venetianer, Biological Research Center, Szeged, Hungary; G. Hager, C/LTVG.

Project Description

Objectives

Fundamental understanding of cellular controls of gene expression remains our prime objective. Studies of steroid hormone action at cellular and molecular levels are being pursued in an effort to achieve this goal. Extension of basic laboratory findings to clinical problems has been possible, and has proved a fruitful secondary objective. In addition to steroid hormones, the action of certain other small molecules known to affect gene expression have been under study.

Specific objectives relevant to the above are as follows:

1) Objective number one has been and remains, to define controls over steroid responsiveness at the cellular level. To this end, cell variants in tissue culture cells have been developed and studied, examining both inductive and inhibitory responses. Particular emphasis has been placed on the development of the CEM tissue culture line of human leukemic cells as a model system for studying the actions of glucocorticoids in human acute lymphoblastic leukemia, including the nature of the events leading to cell death, those responsible for leukemic cell resistance to steroids, and the interactions of drugs used in the treatment of ALL.

Also emphasized has been a study of multiple steroid responses in HTC cells, the rat hepatoma line. Variants of these cells were cloned and chosen for the property of non-inducibility of tyrosine aminotransferase. An objective has been to see whether there was coordinate loss of responsiveness to steroids with respect to a number of other steroid-sensitive processes. The variants were also infected with mouse mammary tumor virus to examine the effects of this steroid inducible virus in these transaminase noninducible cells.

2) Objective two was the purification and characterization of glucocorticoid receptors, comparing their properties with receptors for other steroid hormones. This work has progressed well and continues.

3) Objective three, continuing from last year, is the study of steroid receptors in blood dyscrasias and breast cancer, examining their role in the biology of these diseases and their predictive value in planning therapy.

4) Objective four from last year, to find the nature of mutagenesis and oncogenic transformation in xeroderma pigmentosum cells in vitro, has been terminated due to changes in personnel.

5) Objective five from last year was to examine the translatable levels of tyrosine aminotransferase in tissue culture cells under various conditions of hormone stimulation. Experiments to attain this objective have been temporarily suspended due to limited personnel.

6) Objective six, continuing from last year, has been to set up a translation assay for prolactin mRNA, to isolate this mRNA, prepare its cDNA, and use this reagent to quantify prolactin gene numbers and prolactin mRNA under several conditions of hormonal control in GH cultured cell lines and in pituitary cells.

7) Objective seven, a new one, has been to develop affinity labels for glucocorticoid receptors.

8) Objective eight, also a new one, was to characterize the steroid receptors in and steroid responses of mouse lymphoid cell lines carrying specific Ly surface antigens known to define cells in various stages of functional differentiation.

9) Objective nine is to map: a) the human chromosome carrying the glucocorticoid receptor, and b) the chromosome(s) responsible for the extinction of tyrosine aminotransferase induction in cell hybrids between inducible and non-inducible cells and to see whether the same chromosome carries the information for inhibiting the induction of glutamine synthetase in the same cells.

10) Collaborations with other groups, mentioned in last year's report, all described in detail in the reports of those groups, were translation of A particle RNA in vitro (E. Kuff), translation of AMV RNA in vitro (D. Marciani and T. Papas), and translation of "capped" vs "uncapped" prokaryotic mRNA's in vitro (see project of B. Paterson). Also, another objective which has been completed, was the definition of the ontogeny of glucocorticoid receptors in strains of inbred mice exhibiting high or low teratogenesis following steroid treatment in utero (D. Salomen).

Methods Employed

Most methods have been described in previous reports. Cell methods include somatic cell hybridization; karyotyping by chromosome banding with quinacrine, Giemsa and Hoechst 33258 dye; mutagenesis by physical and chemical agents; cell cloning; growth of tumor cell lines in nude mice.

Biochemical methods include guanidine HCl extraction of cell RNA, translation of mRNA's in the wheat germ system, various types of column chromatography, synthesis of cDNA by AMV reverse transcriptase, restriction

endonuclease cleavage of mixed mRNA's, assays of glutamine synthetase, tyrosine aminotransferase, DNA, and protein; gel electrophoresis, cytoplasmic and whole cell steroid receptor assays, velocity gradient centrifugal analyses of proteins, flow microfluorometry, and isoelectric focusing.

Major Findings

Objective One

a) CEM cells, the tissue culture line of T-cell type human acute lymphoblastic leukemia, have been studied in several ways. First, combined flow microfluorometry and cell cloning experiments have extended our finding of last year that glucocorticoids trap these cells in the G_1 phase of the cell cycle. We found that the block is irreversible, and cells stopped in G_1 appear to be those which go on to die. Second, studies of the combined effects of steroids and other antitumor drugs have shown that while prednisolone and vincristine or methotrexate are additive or slightly synergistic, the steroid and 6-mercaptopurine together are antagonists. Third, fluctuation analysis of the development of resistance to glucocorticoids has shown that CEM cells spontaneously develop resistance by independent events occurring at a rate of about one per 10^5 cells per generation. Some 60 independent spontaneous resistant clones have been isolated and analyzed for the presence of glucocorticoid receptors. All but one possess measurable quantities of receptor. In nearly all however, the quantity of receptors/cell is less than normal, as is the sequestration of steroid-receptor complex in the nuclei of the cells. Thus, in these human leukemia cells, in contrast to earlier studies on mouse lymphoid cells, the commonest alteration accompanying the development of steroid resistance appears to be, not loss of receptor, but a reduction in receptor numbers together with reduced ability of steroid-receptor complexes to translocate from cytoplasm to nucleus. Fourth, glutamine synthetase was discovered to be stimulated in CEM cells by glucocorticoids. Its induction was lost in all the resistant clones, and it therefore should prove a useful marker of steroid sensitivity in these cells. Fifth, the growth of CEM cells has been found to be inhibited by 25 hydroxy cholesterol, and preliminary identification of a binding protein for this hydroxylated sterol has been made in CEM cell cytoplasm.

b) HTC cell variants. Three clones of HTC rat hepatoma cells were arbitrarily chosen from a number of clones found to possess basal tyrosine aminotransferase which could not be induced by glucocorticoids. Five other functions sensitive to glucocorticoids in these cells were then examined: inhibition of cyclic AMP phosphodiesterase, of plasminogen activator, and of uptake of α-amino isobutyric acid; and induction of tyrosine aminotransferase by dibutyryl cyclic AMP following steroid treatment and of glutamine synthetase. There was not a coordinate loss of all five functions in the tyrosine aminotransferase non-inducible clones, but all three clones showed the same pattern. All were resistant to steroids with respect to the dibutyryl cyclic AMP effect and phosphodiesterase suppression. All responded normally with respect to plasminogen activator suppression and

glutamine synthetase induction. And all showed inhibition of α-amino AIB uptake, albeit with somewhat slower kinetics and to a somewhat lower extent than wild-type cells. These results imply a multiplicity of specific intracellular controls which define the particular group(s) of steroid responses in a particular cell.

c) Clones of steroid-resistant L cells (mouse fibroblasts) have been obtained with apparently normal glucocorticoid receptors.

d) Last year we had found that hybrids between GH_3 cells (prolactin +, induced by TRH, and estrogen) and L cells (prolactin -) were prolactin - and non-inducible. One clone of the hybrids later became a high constitutive producer of prolactin. Karyological analysis of this clone now show it to have segregated all identifiable L cell chromosomes and to have lost part of one GH_3 chromosome.

Objective Two

The two forms of glucocorticoid-receptor steroid complex identified last year have been further studied. We believe them to represent the pre-activated and activated forms of the complex. Activation can be achieved by heat, dilution, exposure to 0.3 M KCl, or by precipitation with $(NH_4)_2SO_4$. The pH optimum of activation is 8.0. The activated form binds to chromatin, DNA or nuclei; the pre-activated form binds well to none of these. Preliminary data suggests that the activated form exists in two slightly different sizes.

Objective Three

The role of steroid receptors in leukemia and breast cancer has been further defined. In Sézary's syndrome, a rare form of erythrodermic luekemia whose cells have suppressor T-cell properties, glucocorticoid receptors have been identified and characterized.

Data on estrogen, androgen, glucocorticoid and progestin receptors from several hundred breast cancer patients have been analyzed, and several important conclusions reached. First, lack of estrogen/receptor predicts a far better response to chemotherapy than that obtained in patients whose tumor cells possess such receptors. Second, estrogen receptors appear to represent a prognostic factor, independent of other factors, which predict a longer disease-free interval and better course for patients whose tumors possess them. Third, our data in male breast cancer patients show the tumors of nearly all to contain estrogen receptors, but these receptors appear to have less predictive value than in women with breast cancer. Fourth, benign breast lesions contain estrogen receptors less often than do cancers. Fifth, all combinations of receptors may be seen in breast cancer, but estrogen receptor positive tumors are more often positive for other receptor types. The combined presence of ER and PR, in confirmation of other recent work, predict patients who will respond to endocrine therapy better than does ER alone.

Objective Six

Prolactin mRNA. Using the constitutive prolactin-producing clone of GH_3 x L cell hybrids, we have extracted total cell RNA, isolated total mRNA, and shown it to translate into immunoprecipitable prolactin in the wheat germ system. Estimates of the rate of prolactin production in these cells, by pulse labeling with amino acids and immunoprecipitation give a rate of about 6% of total protein production as prolactin. In the wheat germ system, about 8% of the protein product is prolactin. We have prepared cDNA from the total mRNA population.

Objective Seven

Affinity labels for glucocorticoid receptors are being developed. Several appear promising, both conventional and novel types. Some have been shown to be agonist-antagonists of tyrosine aminotransferase in HTC and Fu55 cells, to compete for binding to glucocorticoid receptors in vitro, and to prevent exchange of radiolabeled ligands onto receptors in vitro.

Objective Eight

Glucocorticoid receptors have been identified, quantified, and characterized for T cells with helper, suppressor or neither function. The suppressor cells' function has been shown to be blocked by concentrations of dexamethasone which saturate these receptors. This, and the work with Sézary cells, is the first characterization of GR in specialized subsets of T-related lymphoid cells and the first evidence of a direct steroid effect on such cells.

Proposed Course of Research

We plan to pursue the work on CEM cells along several lines. We will continue to study the nature of the resistant clones, to see whether they are in fact receptor mutants, whether they contain dominant factors which confer steroid resistance in cell hybrids, and whether conditions can be found which render them sensitive to steroids. Other resistant lines will be developed after mutagenesis of the sensitive parent, and if resistant clones with different properties can be obtained, complementation studies by somatic cell hybridization will be carried out. We intend to test the theory that steroids kill cells by inducing special RNA(s) through RNA-DNA annealing experiments. We will continue our current experiments attempting to map the chromosome bearing the glucocorticoid receptor by use of mouse-human hybrids.

We plan to see whether the HTC non-inducible variants produce translatable tyrosine aminotransferase mRNA.

We intend to continue to accumulate receptor data in the leukemias and breast cancer. In many leukemias it seems that most cells contain receptors

even if they are resistant to steroids. Recently, various cell markers have allowed more perceptive classification of leukemias than was formerly possible when classification was by cytology only. Therefore we want to correlate receptor content with other markers of leukemic cells. In both both leukemia and breast cancer we want to follow patients on whom data is obtained to look for correlations between receptors and the natural history of the diseases.

With the prolactin-producing cells, we want to subject the total cDNA to restriction endonucleases and then electrophorese the resulting digest. This will be done both on the original cDNA product, which presumably is mostly single stranded, and on a double stranded cDNA, prepared by treating the single stranded with DNA polymerase (Pol I). If, as we hope, this procedure produces one or a few unique classes of cDNA restriction fragments, we will try to identify it (them) as prolactin-specific by both direct sequencing and by hybrid arrest interference with prolactin mRNA translation in vitro. If specific prolactin mRNA fragments are not achieved from total mRNA, we will carry out some initial purification steps before restricting. Once prolactin cDNA is identified, we intend to use it for purification of prolactin mRNA, prolactin DNA, and for physiological studies of prolactin mRNA under various hormone treatments.

Affinity labeling studies of glucocorticoid receptor will be pursued by obtaining radiolabeled forms of the more promising ligands and directly testing for covalent binding to receptor. Other affinity label compounds will also be developed.

In the lymphoma lines bearing Ly antigens the interactions of steroids which result in cell killing will be investigated in detail, since such synergism has considerable potential interest therapeutically.

Gene mapping of glucocorticoid receptor and of the putative inhibitor of tyrosine transaminase induction will be continued by karyological analysis of appropriate hybrids.

Publications

Granner, D. K., Lee, A., and Thompson, E. B. Interaction of glucocorticoid hormones and cyclic nucleotides in induction of tyrosine aminotransferase in cultured hepatoma cells. J. Biol. Chem., 252:3891-3897, 1977.

Gopalakrishnan, T. V., and Thompson, E. B. The stability of tyrosine aminotransferase and other proteins in enucleated rat hepatoma tissue culture cells. J. Cell. Physiol., 93:69-79, 1977.

Norman, M. R., and Thompson, E. B. Characterization of a glucocorticoid-sensitive human lymphoid cell line. Cancer Res., 37:3785-3791, 1977.

Sakaue, Y., and Thompson, E. B. Characterization of two forms of glucocorticoid hormone-receptor complex separated by DEAE-cellulose column chromatography. Biochem. Biophys. Res. Commun., 77:533-541, 1977.

Thompson, E. B., Norman, M. R., and Lippman, M. E. Steroid hormone actions in tissue culture cells and cell hybrids--their relation to human malignancies. Rec. Prog. Horm. Res., 33:571-615, 1977.

Salomon, D. S., Zubairi, Y., and Thompson, E. B. Ontogeny and biochemical properties of glucocorticoid receptors in mid-gestation mouse embryos. J. Steroid Biochem., 9:95-107, 1978.

Lippman, M. E., Allegra, J. C., Thompson, E. B. et al. The relation between estrogen receptors and response rate to cytotoxic chemotherapy in metatastic breast cancer. New Eng. J. Med., 298 (No. 22):1223-1228, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB05245-01 LB

PERIOD COVERED October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Molecular Studies of Viral and Eukaryotic Patterns of Transcription

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Bruce M. Paterson Staff Fellow LB NCI

COOPERATING UNITS (if any)

Martin Rosenberg, C/LMB; Bryan E. Roberts, Brandeis University;
Michael B. Mathews, Cold Spring Harbor Laboratory.

LAB/BRANCH Laboratory of Biochemistry

SECTION Biochemistry of Gene Expression Section

INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	2	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The regulation of gene expression in eukaryotic cells has been studied in two different systems. Using nucleic acid hybridization techniques, earlier work provided a detailed analysis of the mRNA population in differentiating muscle cell cultures and demonstrated changes in coding capacity, complexity and abundance class distributions during myogenesis. Adenovirus type-2 infected HeLa cells have been used to study the regulation of viral gene expression. Methods to map the viral structural genes were developed and have been combined with new techniques for selecting and sizing viral specific mRNA transcripts. This has given an overview of the structural gene arrangement and transcription pattern within the viral genome. The structure of mRNA has been studied with regard to those parameters which modulate translational efficiency, utilizing defined prokaryotic mRNA transcripts, modified structurally in various ways, in eukaryotic cell-free translational systems. Eukaryotic ribosome binding sites on these prokaryotic mRNAs have been sequenced and compared to prokaryotic ribosome binding sites on the same mRNA, characterizing the common mRNA structure seen by both types of ribosomes.

Objectives

Work is directed towards the elucidation of gene regulation in eukaryotic cells. The DNA animal viruses provide some of the better systems for the study of gene regulation in eukaryotic cells since the viral genome and viral gene products are well defined and are regulated, in part, by mechanisms which operate in the host cell. Adenovirus type-2 infected HeLa cells provide the model system for further studies. We are currently defining the structural gene map for the early and late viral functions and are correlating each viral protein with its particular mRNA transcript. The size of a particular transcript helps to define overlap arrangements once the coding information within a particular DNA fragment of the viral genome is defined. This same RNA is used to screen the genome for the positions of sequence homology in order to clarify gene splicing patterns and to reconfirm the structural gene arrangement. This information will be used to generate the transcription and processing scheme used to regulate the expression of viral information. Work will also continue to explore aspects of mRNA structure in relation to its translation in eukaryotic and prokaryotic systems.

Methods

The aforementioned objectives involve the use of standard RNA and DNA isolation proceedures, preparative isolation of various restriction fragments of the viral DNA, structural gene mapping by hybrid arrested cell-free translation, mRNA selection techniques using DNA bound to cellulose, isolation of R-looped mRNAs on agarose columns, Southern blots of 32-P labeled selected mRNA to various restriction fragments of viral DNA, sizing of mRNAs on methylmercury agarose gels, cell-free translation of the sized mRNAs in message dependent rabbit reticulocyte lysates or wheat germ extracts, S-1 mapping of the sized mRNAs.

Major Findings

We have been able to define the structural gene map for the late adenovirus proteins. We have determined the sizes of the majority of the late mRNA transcripts and have preliminary data on the major early transcripts. This information has enabled us to define a partial mRNA map which reveals regions of mRNA overlap and defines those regions of mRNA which encode structural information. This has been related to RNA processing and splicing.

With regard to studies on mRNA structure, addition of a cap structure, or 7-methyl G moiety, to the 5' end of a prokaryotic mRNA enables eukaryotic protein translational systems to synthesize specific prokaryotic proteins as efficiently as eukaryotic proteins. Studies on ribosome binding sites demonstrate that both prokaryotic and eukaryotic ribosomes recognize the same ribosome binding sites on prokaryotic mRNAs; thus the cap structure seems to be the major structural difference between prokaryotic and eukaryotic mRNAs.

Projected

We will continue our approach in the analysis of viral gene regulation as described above and will complete the mRNA map for the virus. We will then look at primary transcription and study initiation and processing in detail. Early viral functions present a complex picture at the present time but we intend to analyze this aspect of viral infection in detail since the transformation functions of the virus are early events in infection.

Studies on mRNA structure will continue. We intend to analyze the role of the cap structure in translation by generating mRNA transcripts with varying nucleotide tracts between the cap and the initiation codon. This will give information on position and intervening sequence effects in relation to template efficiency. We are going to develop a system for the analysis of processed prokaryotic mRNAs in the more efficient eukaryotic cell-free systems. This will be used to define and analyze a variety of prokaryotic regulatory proteins that have not been approachable in standard prokaryotic translational systems.

Publications

Paterson, B. M., and Bishop, J. O. Changes in the mRNA population of chick myoblasts during myogenesis in vitro. Cell, 12:751-765, 1977.

Paterson, B. M., Roberts, B. E., and Kuff, E. L. Structural gene identification and mapping by DNA-mRNA hybrid arrested cell-free translation. Proc. Natl. Acad. Sci. USA, 74:4370-43, 1977.

Paterson, B. M., Marciani, D. J., and Papas, T. S. Cell-free synthesis of the precursor polypeptide for avian myeloblastosis virus DNA polymerase. Proc. Natl. Acad. Sci. USA 74:4951-4954, 1977.

Paterson, B. M., Segal, S., Lueders, K., and Kuff, E. L. RNA associated with murine intracisternal type-A particles codes for the main particle protein. J. Virol., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00366-08 LB

PERIOD COVERED October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Biosynthesis and Assembly of Intracellular Components

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E. L. Kuff	Head, Biosynthesis Section	LB NCI
	K. K. Lueders	Chemist	LB NCI
OTHER:	B. Paterson	Staff Fellow	LB NCI
	S. Segal	Visiting Associate	LDNATV NCI
	E. Scolnick	Chief	LTVG NCI

COOPERATING UNITS (if any)

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LAB/BRANCH

Laboratory of Biochemistry

SECTION

Biosynthesis Section

INSTITUTE AND LOCATION

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	2	2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are studying murine intracisternal Type-A particles as a model for the expression of integrated viral genes. A-particle-related genes are present in multiple copies (> 1000 per cell) in cells of Mus musculus. We have demonstrated a nucleotide sequence homology between intracisternal A-particle RNA and the RNA of an extracellular retrovirus derived from the Asian mouse, M. cervicolor.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To study the mechanisms for coordinately regulating the synthesis individual macromolecules and their assembly into complex intracellular components (organelles and viruses); specifically, to study the regulation of endogenous viral gene expression in relationship to normal development and neoplastic transformation.

Methods Employed: Tissue culture; isolation of subcellular organelles and viruses; labeling of nucleic acids and proteins in vivo and in cell-free systems; isolation and fractionation of nucleic acids using techniques of affinity chromatography, density gradient centrifugation and gel electrophoresis; estimation of genetic complexity and gene copy number by quantitative nucleic acid hybridization; chromosomal localization of specific DNA sequences by in situ hybridization; chromatographic, immunochemical and electrophoretic analysis of specific proteins.

Results: We have continued the study of murine intracisternal A-particles, obtaining the first evidence of nucleotide sequence homology between these particles and a type of extracellular retrovirus.

1. Cell-free synthesis of the major A-particle structural protein (p73) was directed by a 35S polyadenylated RNA derived from neuroblastoma A-particles. Identity of the in vitro protein product was established by tryptic peptide analysis. A-particles from our standard myeloma source, MOPC-104E, contain only small amounts of 35S RNA; the major poly(A)-RNA in this case is a 29S species, which also codes for synthesis of p73. Preliminary survey of A-particle-associated RNAs from a variety of tumors, using electrophoresis in agarose gels containing methyl mercury hydroxide, shows that 35S RNA (about 8000 nucleotides in length) is always present, but in variable amounts. Each preparation has also shown a characteristic pattern of discrete lower molecular weight components (24 to 32S) which may represent sub-genomic species related to the 35S molecules.

2. We have generated a representative cDNA from the isolated neuroblastoma 35S RNA (using AMV polymerase in an exogenous reaction primed by calf thymus oligodeoxynucleotide fragments). By hybridization analysis, we have found the 35S RNA shares sequences with both a 32S RNA species from neuroblastoma A-particles and the predominant 28S myeloma A-particle RNA mentioned above. Last year, we identified a set of RNA sequences which were much more abundant in A-particle-producing cells than in particle-negative cells and which were associated with A-particles prepared from three different tumor cell types. We have now found that this set of sequences is contained in the isolated 35S, 32S and 28S RNA components. Preliminary evidence indicates that this "abundant class" of sequences may be localized primarily at the 3' ends of the RNA molecules.

3. It was of interest to determine whether sequences represented in the "abundant class" cDNA included those which coded for synthesis of the particle protein. We entertained the possibility that interaction of cDNA with

a homologous region of mRNA might block translation of that region. This consideration led to development of a general method for coding assignment based on hybrid arrest of translation (HART), which is more fully described in the annual report of B. Paterson.

4. Hybridization kinetics show that sequences homologous to the 35S-cDNA are reiterated more than 500 times per haploid genome of Mus musculus (NIH Swiss, BALB/c). If the entire 8000 nucleotide viral genome is indeed reiterated to this extent, A-particle-related information would represent about 0.1% of the total mouse DNA.

5. The A-particle specific RNAs show no sequence homology with the high molecular weight RNAs of mouse mammary tumor virus or a variety of exogenous and endogenous type-C viruses. On the other hand, we have observed a partial nucleotide sequence homology between the high molecular weight (32S and 35S) RNA components of intracisternal A-particles from a neuroblastoma cell line and the 70S RNA fraction from M432, a type of retrovirus endogenous to the Asian mouse Mus cervicolor (Callahan et al., PNAS, 73: 3579, 1976). M432 cDNA was hybridized to the extent of 30% by the A-particle RNAs. The hybrids showed a lower thermal stability (Δt_m 7°) than those formed with homologous RNA. The reaction was commensurate with that found between M432 cDNA and divergent sequences in the musculus genome. The capacity to hybridize M432 cDNA was closely correlated with the concentration of A-particle sequences in the cytoplasmic RNA of several musculus cell types. The major RNA fraction of M432 virus showed a reciprocal partial reaction with the A-particle cDNAs; the virus, which was grown in NIH/3T3 (musculus) cells, also contained a small proportion of apparently authentic A-particle nucleotide sequences. A subset of A-particle sequences seemed to be almost totally lacking in the main M432 RNA. The A-particle cDNAs hybridized extensively with divergent sequences in cervicolor cellular DNA, indicating that this mouse species may contain not only the partially homologous M432 virogene but also a more complete genetic equivalent of the intracisternal A-particle.

Proposed Course of Research: We will attempt to define the A-particle genome more precisely, to ascertain what other protein products it may specify, to determine the arrangement of the reiterated A-particle genetic elements in the DNA of M. musculus, and to study the factors which regulate expression of these elements.

Publications:

Lueders, K.K., and Kuff, E.L.: Sequences associated with intracisternal A-particles are reiterated in the mouse genome. Cell 12: 963-972, 1977.

Paterson, B.M., Roberts, B.E., and Kuff, E.L.: Structural gene identification and mapping by DNA-mRNA hybrid-arrested cell-free translation. Proc. Nat. Acad. Sci. USA 74: 4370-4374, 1977.

Paterson, B.M., Segal, S., Lueders, K.K., Kuff, E.L.: RNA associated with murine intracisternal type-A particles codes for the main particle protein. J. Virology, in press, July 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 CB 00379-17 LB

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Ultrastructural Characterization of Macromolecules

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHER:	R. B. Gennis		Not NIH
	S. Nagasawa		Not NIH
	R. M. Stroud		Not NIH

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Department of Pharmaceutical Sciences, University of Hokkaido, Sapporo, Japan
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5	4	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The enzyme, pyruvate oxidase, has been characterized by negative staining and transmission electron microscopy. The molecule consists of 4 tightly packed subunits and resembles a sphere flattened at both ends. It is 11.5 nm high and 12.4 nm in diameter.

One of the regulators of the complement "cascade", C3b-C4b inactivator co-factor, is a 10^6 dalton protein that closely resembles IgM in its structure.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To relate the fine structure of macromolecules to their biochemical and physical properties, and to their function in biological systems.

Methods and Major Findings:

1. Ultrastructure of pyruvate oxidase: The enzyme is a peripheral membrane flavoprotein of E. coli that mediates the flow of electrons from the water-soluble substrate, pyruvate, to the membrane-bound acceptor, probably cytochrome b_1. The molecule resembles a sphere flattened at both ends, 11.5 nm high and 12.4 nm in diameter. The four subunits are longer than they are wide and are closely packed so that the negative stain tends to be excluded from the space between them. The subunits are prolate spheroids with major and minor axes of 12.4 and 3.4 nm, respectively. Given the partial specific volume of 0.75 cm^3/gm, the molecular weight of the subunit calculates to be 59,887 daltons. This is compatible with the molecular weight of 60,000 calculated by equilibrium centrifugation.

2. Ultrastructure of C3b-C4b activator co-factor: This co-factor regulates the recruitment of C3 and C4 into the chain of events leading to the attack by complement on the cell membrane. The ultrastructure of the co-factor strongly resembles IgM in having five arms extending from a central core. The structure is compatible with the molecular weight, 10^6 daltons, the same as IgM.

Significance to Cancer Research: A knowledge of the structure of macromolecules obtained by observations with the electron microscope provides confirmation of physico-chemical data and, in many cases, an unequivocal basis for knowledge of their function.

Proposed Course of Research: Terminated.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 CB 00945-05 LB

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Study of the Factors Regulating the Synthesis of Collagen in Normal and Transformed Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B. Peterkofsky	Research Chemist	LB NCI
OTHER:	J. H. Eichhorn	Research Associate	LB NCI
	J. Mata	Visiting Associate	LB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Biosynthesis Section

INSTITUTE AND LOCATION

DCBD, NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5	3	2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Our studies are concerned with various aspects of collagen metabolism. Prolyl and lysyl hydroxylases, enzymes which catalyze the hydroxylation of prolyl and lysyl residues in collagen during synthesis of this protein on polysomes bound to the membrane of the endoplasmic reticulum, require Fe^{++}, α-ketoglutarate, oxygen and a reducing compound. Ascorbic acid is the most efficient reducing compound but others such as tetrahydropterins, tetrahydrofolate, dithiothreitol and cysteine are also active to varying extents. The localization of these enzymes in microsomes as well as the specificity of the reducing compound are being studied. Since hydroxylation of prolyl residues in collagen is required for collagen secretion while hydroxylysine is required for the formation of crosslinks to form mature collagen fibers, the enzymes and their cofactors may play a role in the regulation of collagen synthesis and the maintenance of connective tissue. Collagen synthesis is also being used as a marker to study the effects of neoplastic transformation on a differentiated cell function.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To elucidate the basic mechanisms of collagen synthesis and secretion and to investigate the factors which regulate these processes in normal and transformed cells.

Methods Employed:

1. Proteins of whole chick embryos, isolated bones of these embryos, or cell-free systems derived from these bones or cultured fibroblasts are labeled with ^{14}C-proline. The proteins are precipitated with trichloroacetic acid, redissolved and then assayed for radioactivity in collagen and non-collagenous proteins by a method involving specific digestion of collagen in a mixture of proteins using highly purified collagenase. This method was developed in our laboratory and is described in a previous report (1968-9). The relative rate of collagen synthesis can be calculated from data obtained by this method and the specific rate of synthesis in tissue or cells is calculated by determining the DNA content and expressing the rate as cpm/μg DNA. The proteins of the cell and medium fractions of cultured fibroblasts are analyzed separately in order to study secretion. Morphological changes in cultured cells are recorded by polaroid photomicroscopy.

2. Cell-free incorporation of ^{14}C-proline is studied with a system consisting of membrane-bound or free polysomes derived from chick embryo bone tissue and soluble factors derived from chick embryo liver, a tissue which synthesizes very little collagen, so that variations in collagen synthesizing polysomes of a tissue may be studied independently of possible variations in the soluble fraction.

3. To determine the level of proline hydroxylation in collagen of cells or tissues labeled with radioactive proline, collagenase digests are hydrolyzed with 6N HCl and radioactive proline and hydroxyproline in the hydrolyzates are measured by a specific radioassay. To measure the level of lysine hydroxylation, cells or tissues are labeled with radioactive lysine and hydrolyzed collagenase digests are chromatographed on Dowex-50 in 2N HCl, which separates lysine and hydroxylysine.

4. Prolyl and lysyl hydroxylases are measured by $^{3}H_2O$ release from ^{3}H-proline or ^{3}H-lysine labeled unhydroxylated collagen prepared by incubating chick embryo frontal bones with the labeled amino acid in the presence of the iron chelator α,α'-dipyridyl, which inhibits hydroxylation.

5. Tetrahydropteridines in cell extracts were measured using purified phenylalanine hydroxylase which has a specific requirement for this cofactor.

6. Collagen types synthesized in cultured cells were determined by analysis of ^{14}C-or ^{3}H-proline labeled, denatured collagen using carboxymethyl cellulose chromatography and gel electrophoresis in sodium dodecyl sulfate-polyacrylamide gels. The presence of disulfide bonds was detected by running samples with and without dithiothreitol and observing alterations in the positions of α chains.

Cell Lines used in these studies: Chick embryo fibroblasts: prepared by digesting frontal bones from 15 day chick embryos with crude collagenase and culturing the released cells. Subcultures in the second to fifth passage are used.

L-929- a line of mouse embryo fibroblasts established about 30 years ago by use of a chemical carcinogen.

BALB-3T3- a contact inhibited line of mouse embryo fibroblasts isolated by Todaro and Aaronson. We have derived several subclones of this line: P3, which is more stringently contact-inhibited than the original culture and P13, which has lost density dependence.

BALB-3T6 and 3T12- lines of mouse embryo fibroblasts derived from the same pool of cells as 3T3 but non-contact inhibited and tumorigenic.

Ki-3T3- a line established by Aaronson by transformation of BALB 3T3 cells with Kirsten sarcoma virus. These cells do not produce viral particles unless super-infected with murine leukemia virus.

Mo-3T3- BALB 3T3 cells transformed with Moloney sarcoma virus.
SV-3T3- BALB 3T3 cells transformed with SV-40 virus.
NQT-3T3- BALB 3T3 cells transformed with a chemical carcinogen, 4-Nitroquinoline-1-oxide.

Embryonic and adult human diploid fibroblasts-purchased commercially.

Major Findings:

I. Submicrosomal localization of lysyl hydroxylase.

We found that approximately 30% of lysyl hydroxylase activity in limb bone microsomes occurs in the cisternae which corresponds to the lumen of the endoplasmic reticulum. This conclusion was based on the observations that this fraction of enzyme activity could be solubilized by low concentrations of nonionic detergents and was resistant to mild trypsin proteolysis. The remainder of the activity in the microsomes could only be extracted by a combination of detergent plus high ionic strength (0.5 M NaCl or KCl), suggesting that the tightly-bound enzyme is associated with the microsomal membrane through ionic bonds. The microsomal tightly-bound enzyme activity was partially inactivated by mild trypsin proteolysis but inactivation was greatly increased by carrying out the trypsin treatment in the presence of 0.05% Brij, which allows access of the trypsin to the microsomal cisternae. These results and others led us to the conclusion that the tightly-bound enzyme is a transmembrane protein with its active site on the cisternal surface of the membrane.

II. The role of ascorbic acid in collagen metabolism.

A. Identity of a reducing cofactor for prolyl hydroxylase in cultured cells.

We have shown that cell-free extracts derived from stationary phase L-929 and Kirsten sarcoma virus transformed BALB 3T3 (Ki-3T3) cells, which exhibit ascorbate-independent proline hydroxylation, contain a reducing cofactor which can substitute for ascorbate in an in vitro assay devised to measure cofactor activity. This substance is not ascorbate or any of the other reducing agents which can partially replace ascorbate as cofactor, such as tetrahydropterin, tetrahydrofolate or cysteine. In contrast, log phase L-929 and normal BALB 3T3 cells, both of which require ascorbate for maximum proline hydroxylation, contained little (L-929) or none (3T3) of the endogenous reducing substance. These results suggested that the reducing cofactor measured in vitro could account for the cellular phenomenon of ascorbate-independent proline hydroxylation but more direct evidence was required.

Since prolyl hydroxylase is localized in the cisternae of the microsomes, one approach has been to fractionate subcellular organelles of stationary phase L-929 and Ki-3T3 cells in order to determine whether the cofactor is also localized in microsomes. This would be a prerequisite if the enzyme and cofactor are to function together. Preliminary experiments have eliminated nuclei and mitochondria as possible sites for the cofactor and further improvement in fractionation techniques will be required to differentiate between microsomes, cell membranes and lysosomes.

Another approach has been to find a correlation between prolyl hydroxylase activity and the level of cofactor in cells. It has been previously shown that culture conditions which favor glycolytic metabolism lead to activation of prolyl hydroxylase and we have proposed that such conditions may also lead to increased production of or a higher reduction level of the endogenous cofactor and that this in turn may lead to prolyl hydroxylase activation. By growing Ki-3T3 cells in the presence of 5 mM pyruvate and 1/10 the normal amount of glucose, glycolysis, as measured by lactate production, was reduced to about 1/3 of control cells. There was, however, no effect on ascorbate-independence of collagen secretion or proline hydroxylation and the levels of prolyl hydroxylase or endogenous reducing cofactor did not decrease. These results suggest that the relationship of the metabolism of these cells to various parameters of collagen metabolism are more complex than anticipated and it is possible that merely decreasing lactate production does not necessarily alter the pathway of glucose oxidation.

III. Effect of Local anesthetics on collagen secretion.

Because of our interest in the role of microtubules in collagen secretion and recent reports suggesting that local anesthetics affect microtubule function, we examined the effects of these agents on collagen secretion. The local anesthetics procaine, tetracaine, lidocaine, mepivicaine, dibucaine and cocaine inhibited collagen secretion in BALB 3T3 cells and diploid human fibroblasts as much as 98%. These agents were more effective than colchicine, a known microtubule disrupter, which produced a maximum of 50% inhibition. When cells were treated with subsaturating concentrations of colchicine and procaine, the inhibition of secretion was an exact sum of the inhibition by

each drug individually, suggesting that the microtubule system may be the target of both. The effects of the local anesthetics and colchicine on morphology were quite different, however, and unlike colchicine, the local anesthetics did not bind to purified tubulin in vitro or cause depolymerization of microtubules when 3T3 cells were incubated with the drugs. These experiments have led to the conclusion that local anesthetics may interfere with microtubule function but must do so by a mechanism which does not lead to depolymerization.

Proposed Course of Research:

I. Lysyl hydroxylase.

It will be of great interest to determine why the microsomal localization of prolyl hydroxylase, which is mainly intracisternal, and of lysyl hydroxylase, which is mainly transmembrane with about 30% intracisternal, is so vastly different. Both enzymes carry out similar reactions which require identical cofactors and function during synthesis of procollagen chains on membrane-bound polysomes. To this end, the properties of the lysyl hydroxylases from the two different sites will be compared with respect to cofactor and substrate requirements, effect of inhibitors, molecular weights and heat stability. Since some residues of hydroxylysine in collagen are further modified by glycosylation with UDP-glucose and UDP-galactose to form galactosyl-hydroxylysine and glucosyl, galactosyl-hydroxylysine, while hydroxyproline is not modified, the possibility exists that this is the basis for the different localization of the two enzymes. Therefore, the spatial relationship between lysyl hydroxylase and the two glycosylating enzymes, which also occur in microsomes, will be examined.

II. Reducing cofactor in cultured cells.

Further improvement in fractionation of subcellular particles will be made in order to more accurately localize the cofactor. As another approach, experiments using intact microsomes containing endogenous prolyl hydroxylase will be carried out to determine whether the cofactor can function in proline hydroxylation under such conditions. If so, this would provide strong evidence that the cofactor and enzyme function together in cells.

III. Modification of the collagen phenotype by transformation.

Methods will be devised to make detection of type III collagen simpler so that additional transformed lines can be screened to determine whether this collagen type specifically appears in sarcoma virus transformants.

Significance to Biomedical Research and the Program of the Institute:
Determining the precise mechanism by which vitamin C affects collagen synthesis should lead to a more accurate use of this vitamin. In addition, our studies with transformed cells indicate that, at least under some conditions, animal cells may be able to synthesize a substitute for the function of vitamin C in connective tissue.

Our studies with dbcAMP are aimed at determining the extent to which this chemical reverses cell transformation.

Publications:

Hata, R., and Peterkofsky, B.: Specific changes in the collagen phenotype of BALB 3T3 cells as a result of transformation by sarcoma viruses or a chemical carcinogen. Proc. Nat. Acad. Sci. USA 74: 2933-2937, 1977.

Hata, R., and Peterkofsky, B.: Retention of transformant specific type III collagen in dibutyrylcAMP treated Kirsten sarcoma virus transformed BALB 3T3 cells in a flat revertant. J. Cell. Physiol. 95, 343-352, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB05214-07 LB

PERIOD COVERED October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

DNA Synthesis in Mammalian Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. H. Wilson	Medical Officer	LB NCI
OTHER:	Y.-C. Chen	Visiting Fellow	LB NCI
	S. Planck	Guest Worker	LB NCI
	K. Tanabe	Visiting Fellow	LB NCI
	B. Carter	Senior Investigator	LEP NIAMDD

COOPERATING UNITS (if any)

Laboratory of Experimental Pathology, NIAMDD

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Biosynthesis Section

INSTITUTE AND LOCATION

DCBD, NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5	4	1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Several mouse myeloma proteins important for analysis of in vitro DNA replication have been purified to near homogeneity. These include DNA polymerases α and β and DNA unwinding protein. The native molecular weight of α-polymerase is about 300,000, and it contains at least four subunits; the β-polymerase is one polypeptide chain of 40,000 M.W.; DNA unwinding protein is one polypeptide chain of about 25,000 M.W., however, this protein exhibits microheterogeneity upon isoelectric focusing or high resolution SDS-polyacrylamide gel electrophoresis. Under certain conditions DNA unwinding protein decreases the Tm of poly d(A-T) by 25°. Controlled proteolysis of DNA unwinding protein results in a discrete fragment that is similar to the intact molecule in its avid binding to single-stranded DNA, but different in that it does not decrease the Tm of DNA. Steady state kinetic studies of β-polymerase have provided data that point to a sequential mechanism for DNA formation. Magnesium activation of this reaction does not change the Km for either dNTP or template-primer, suggesting that Mg^{2+} and the substrates bind independently to the enzyme and that Mg^{2+} does not seriously affect binding of the substrates.

PHS-6040
(Rev. 10-76)

Project Description:

Objective: The objective of this research program is to understand mechanisms involved in DNA synthesis in mammalian cells. Our approach is the examination of DNA replication in vitro using purified DNA polymerases and other required proteins.

Previous analysis in this laboratory of the DNA polymerases of mouse myeloma revealed three enzymes that are analogous to DNA polymerases well known in a variety of mammalian tissues. We have now succeeded in purifying these enzymes to near homogeneity and are involved in rigorous characterization of their molecular and biochemical properties. We hope to extend these studies to an analysis of a portion of the replication cycle of a defined genome from mouse cells.

It is anticipated that these studies on the properties and specificities of the purified DNA replication proteins will, in conjunction with results from other workers, help answer important question about mechanisms of DNA replication in the cell.

Methods Employed: Procedures have been developed for purification of DNA polymerases and DNA binding-protein from mouse tissue. These procedures involve subcellular fractionation, various types of ion exchange, gel filtration, and affinity chromatography, and assays for DNA polymerase activities using a variety of reaction conditions. Cell fractionation procedures and characterizations of reaction products were performed using conventional techniques of rate-zonal centrifugation and scintillation spectrophotometry.

Major Findings:

1. Characterization of near homogeneous DNA polymerase alpha:

We have examined the subunit structure of mouse DNA polymerase alpha. The enzyme was purified to near homogeneity from the soluble protein fraction of mouse myeloma using two distinctly different methods that had recently been developed in this laboratory. As revealed by SDS-polyacrylamide gel eletrophoresis, the "pure" enzyme obtained by each method contained four main polypeptides. The apparent molecular weights of these polypeptides in the two enzyme preparations were the same. Based upon this and other evidence of cofractionation of these four polypeptides and the enzyme activity, we believe that our near homogeneous mouse alpha-polymerase is an oligomeric protein containing at least four subunits. The native enzyme has a molecular weight of about 300,000 as determined by Sephacryl S-200 gel filtration.

2. Characterization of near homogenous DNA polymerase beta:

DNA polymerase beta was extracted from a nuclear fraction of mouse myeloma and then purified using a modification of a procedure described by Chang (J. Biol. Chem. 248: 3789, 1973). The native enzyme has a molecular weight of about 40,000 by gel filtration. The final enzyme preparation contained

essentially one polypeptide as determined by SDS-polyacrylamide gel eletrophresis, and this had an apparent molecular weight of 40,000. Steady state kinetic studies of the DNA formation reaction provided data that point to a sequential mechanism. Magnesium activation of the reaction does not change the Km for either dNTP or template-primer, suggesting that Mg^{2+} and the substrates bind independently to the enzyme and that Mg^{2+} does not seriously affect binding of the substrates.

3. Characterization of DNA binding protein:

Using a modification of the procedure described by Herrick and Alberts (J. Biol. Chem. 251: 2124, 1976) we have isolated myeloma DNA binding protein-operationally defined as a class of relatively low molecular weight ($\sim$ 25,000) proteins that pass through a column of double-stranded DNA-cellulose, but are retained on single-stranded DNA-cellulose and remain bound during a rinse with 0.5 mg/ml dextran sulfate or 0.1 M NaCl; the proteins are removed from the column by higher concentrations of NaCl. As in the case of calf thymus, the myeloma DNA binding protein is abundant in the cell, comprising about 1% of total cell protein. Mouse DNA binding protein protein is capable of "unwinding" DNA, since we have found that it lowers the Tm of poly d(A-T) by as much as 25°C depending upon the protein: DNA ratio of the solution. Also, this protein stimulates by 2 to 3 fold the replication of poly d(A) by DNA polymerase α. Thus, this DNA binding protein has interesting "biological activity".

Isoelectric focusing and high resolution polyacrylamide gel electrophoresis have shown that our preparation of DNA binding protein is heterogeneous with regard to both isoelectric point and apparent molecular weight. Tryptic peptide mapping showed that the individual proteins resolved by gel electrophoresis share extensive sequence homology. We have managed to obtain several of the individual species in pure form and have found examples of proteins that bind tightly to single-stranded DNA but do not lower the Tm of double-stranded DNA. A similar picture emerged from experiments using controlled proteolysis of the standard DNA binding proteins. A distinct 23,000 molecular weight fragment was obtained that binds tightly to single-stranded DNA, but does not lower the Tm of double-stranded DNA.

4. Regulation of DNA polymerase alpha in monkey cells infected with adenovirus:

In collaboration with B. Carter and his colleagues, we have studied regulation of alpha-polymerase activity in a line of contact inhibited monkey cells in culture. Our evidence to date suggests that DNA polymerase alpha activity is about 60-fold higher after infection with adenovirus than in mock infected cells. The amount of total cell protein did not change appreciably during the period of the experiment, nor did the amount of DNA polymerase beta activity. If these results indeed reflect the activity and metabolism of alpha-polymerase, adenovirus infection may represent one of the most striking inductions of this enzyme thus far observed.

Significance to Cancer Research: Detail knowledge of the mechanisms of DNA synthesis is vital to our understanding the molecular biology of neoplasia. Many approaches are being used to investigate DNA synthesis in normal and neoplastic cells, and advances on all levels will prove useful in preventing, treating, and ultimately controlling cancer. Activity of DNA synthesizing enzymes is necessary for maintenance of a rapid rate of cell division, and in some cases there is evidence that these proteins may play an important role in the development of the neoplastic state.

Proposed Course of Research: The objective of this research is to understand mechanisms of DNA synthesis in mammalian cells; the main approach is the investigation of the mechanism of action DNA synthesizing proteins in vitro.

1. To characterize DNA polymerases alpha, beta and gamma of mouse myeloma.

2. To characterize DNA binding proteins of mouse myeloma.

3. To investigate the in vitro activity of purified DNA replication proteins using 1) polyoma virus replication intermediates and 2) a single-stranded closed circular bateriophage DNA.

Publications:

Wilson, S.H., Matsukage, A., Bohn, E.W., Sivarajan, M., and Chen, Y.C.: Polynucleotide recognition by DNA α-polymerase. Nucleic Acids Res. 4: 3981-3996, 1977.

Pitha, J., Wilson, S.H., and Pitha, P.M. : A vinyl polymer with purine residues deficient in base pairing inhibits murine leukemia virus replication. Biochem. Biophys. Res. Com. 81: 217-223, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB05215-07 LB

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Biological and Ultrastructural Studies on Cell Cooperation in the Immune Response

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Emma Shelton	Research Biologist	LB NCI
OTHER:	Beverly H. Porter	Expert	LB NCI
	Laird D. Madison	Biological Aide	LB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Biosynthesis Section

INSTITUTE AND LOCATION

DCBD, NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	2	5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Compounds that "uncouple" oxidative phosphorylation, viz., NaN_3, oligomycin and dinitrophenol, cause microtubules to polymerize in the cytoplasm and nucleus of mast cells but not macrophages and lymphocytes.

The cortex of certain cells and the microvilli protruding from it appear to to supported and maintained by a network of fibers consisting principally of actin. The density of microvilli on omental mesothelial cells can be increased or decreased by raising or lowering the ambient protein concentration. Certain types of proteins are more efficient effectors than others. Pits, 150-400 nM in diameter, appear on the cell surface after exposure to protein. The pits, which follow kinetics similar to microvilli, are most frequently found at the base of a microvillus.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To examine the interaction of lymphocytes and macrophages cooperating in the immune response; specifically, to examine the factors controlling the surface morphology of these cells.

Methods and Major Findings:

Mast Cell Microtubules

Sodium azide, 10^{-2} M, causes reversible polymerization of microtubules in mast cell cytoplasmic folds and in the nucleus. NaN has two major metabolic effects; it uncouples oxidative phosphorylation and depletes the ATP pool, and it raises cGMP levels in many, but not all, cells. We tested other compounds for their effect on mast cell microtubules.

Oligomycin, 10 µg/ml and dinitrophenol, 10^{-3} M, both "uncouplers", cause irreversible microtubule polymerization in mast cell cytoplasm and nucleus.

Sodium nitroprusside, 10^{-2} M, a compound that raises cGMP levels, has no effect on mast cell microtubules.

Internal structure of cortex and microvilli of peritoneal macrophages and lymphocytes

Treatment of macrophages and lymphocytes with S-1 fragment of heavy meromyosin or with peroxidase-coupled anti-actin shows the cortical layer and microvilli to be much more densely labeled in cells attached to a substrate than in cells in suspension. This band of actin is greatly reduced when cells are treated with diamide, a compound that eliminates microvilli, and is accentuated when cells are treated with azide, a compound that increases microvillus length. No difference could be detected in the total polymerized actin extracted from normal and azide treated cells. The evidence is strong that the structure of the cytoplasmic cortex and microvilli of these cells is supported by a filamentous network consisting primarily of a meshwork of actin.

Regulation of numbers of microvilli on mesothelial cells

The mesothelial cells covering the omentum of the mouse are sparsely covered with microvilli, ca. 50/um^2. This density can be manipulated according to the concentration and type of protein to which the cells are exposed. For example, intraperitoneal injection of 0.5 ml of normal mouse serum raises microvillus density at 2 minutes (earliest point measured) and continues to a peak of ca. 300/um^2 at 30 minutes then falls to almost normal after 1.5 hours. The kinetics of the response to a similar concentration of bovine serum albumin is quite different. Microvillus density is lower than normal for the first 15 minutes, then rises slowly to a peak of ca. 150/um^2 at 1 hour then returns to a lower level at 90 minutes. Concomitant with the microvillus response is the appearance of 150-400 nM diameter pits in the cell surface. The pits appear most frequently to be located at the base of microvilli.

Proposed Course: Terminated.

Publications:

Orenstein, J.M., and Shelton, E.: Membrane phenomena accompanying erythrophagocytosis: A scanning electron microscope study. Lab. Invest. 36, 363-374, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB05241-03 LB

PERIOD COVERED October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Studies on SV40 Recombinants Containing Cellular and Viral Sequences

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E. L. Kuff	Head, Biosynthesis Section	LB NCI
OTHER:	G. Khoury	Head, Section on Virus Tumor Biology	LDNATV NCI
	F. J. Ferdinand	Visiting Fellow	LDNATV NCI
	E. Winocour	Visiting Scientist	LB NCI

COOPERATING UNITS (if any)

Laboratory of DNA Tumor Virus, NCI
Virology Lab., Weizmann Institute of Science, Rehovot, Israel

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Biosynthesis Section

INSTITUTE AND LOCATION

DCBD, NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have examined the transcription of covalently linked cellular and viral DNA sequences during lytic infection with host substituted variants of SV 40. We have collaborated in a study of the nucleotide sequence of the host substituted DNA. We also report a study showing rescue of an SV40 temperature-sensitive mutant through recombination with SV40 virus endogenous to a transformed monkey cell line.

Project Description:

Objectives: To use the host-substituted variants of SV40 in studying the transcription of viral DNA and the cellular processing of transcripts containing covalently linked host and viral RNA sequences; to understand the process of recombination between SV40 DNA and the DNA of infected cells.

Methods Employed: Cell culture; virus passage; preparation of viral transcriptional complexes; nucleic acid labeling; isolation and hybridization; other virological, biochemical and molecular biological techniques.

Results: The major findings have either been described in previous Annual Reports of this project, or are included in the current report of Project ZO1 CB 5234-05 (Maxine Singer).

With Dr. E. Winocour, Visiting Scientist, we have collaborated in studies to detect primary recombination events between SV40 and monkey cellular DNA, as well as between polyoma and specific cell sequences in the mouse. The results are preliminary and will be described more fully in a subsequent report.

Proposed Course of Research: Under evaluation.

Publications:

Rosenberg, M., Segal, S., Kuff, E.L., and Singer, M.: The nucleotide sequence of repetitive monkey DNA found in defective simian virus 40. Cell 11: 845-857, 1977.

Gluzman, Y., Kuff, E.L., and Winocour, E.: Recombination between endogenous and exogenous simiar virus 40 genes. I. Rescue of a simian virus 40 temperature-sensitive mutant by passage in permissive-transformed monkey lines. J. Virol. 24: 534-540, 1977.

Kuff, E.L., Ferdinand, F.-J., Khoury, G.: Transcription of host-substituted simian virus 40 in whole cells and extracts. J. Virol. 25: 28-36, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB05238-09 LB

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Cloning of Vitellogenin dscDNA and Genomic DNA

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. G. Deeley	Expert	LB NCI
OTHER:	J. I. Gordon	Surgeon	LB NCI
	A. T. H. Burns	Visiting Fellow	LB NCI
	R. F. Goldberger	Medical Director (Chief)	LB NCI
	P. Leder	Medical Director (Chief)	LMG NICHD
	S. M. Tilghman	Visiting Fellow	LMG NICHD

COOPERATING UNITS (if any)

Laboratory of Molecular Genetics, NICHD

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Cellular Regulation Section

INSTITUTE AND LOCATION
DCBD, NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The work described herein consists of a detailed examination by cDNA hybridization of the kinetics of accumulation and disappearance of vitellogenin mRNA in rooster liver following both primary and secondary injections of 17β-estradiol. It has been demonstrated that the enhanced vitellogenic response following secondary treatment with the hormone as compared with that following primary stimulation can be attributed to a more rapid and extensive accumulation of vitellogenin mRNA and does not stem from more efficient translation of the mRNA.

Purified vitellogenin cDNA and mRNA have been used to examine the genomic organization of the vitellogenin gene. The results indicate that the gene may be composed of as many as 25,000 base pairs, while the cytoplasmic form of vitellogenin mRNA is only 7000 nucleotides long. Vitellogenin mRNA has been transcribed into double-stranded cDNA and this DNA has been inserted into the plasmid PBR322 and cloned in E. coli X1776. More than 100 clones containing vitellogenin DNA sequences have been isolated.

Project Description:

Objectives: To elucidate the mechanisms by which estrogen regulates the synthesis of vitellogenin and to examine the genomic organization of the vitellogenin gene.

Methods Employed: Vitellogenin mRNA has been purified from rooster liver total RNA and used as a template for avian myeloblastosis virus reverse transcriptase. The complementary DNA synthesized has been used as a specific hybridization probe to monitor the accumulation of vitellogenin mRNA in rooster liver, following treatment with 17β-estradiol. The concentration of vitellogenin mRNA in liver total RNA has been measured by RNA excess hybridization, at various times after both primary and secondary injections of estrogen. The wheat germ cell-free protein synthesizing system has been utilized to quantitate the amount of translatable vitellogenin mRNA present at similar times during the vitellogenic response.

Chicken DNA has been digested with the restriction endonuclease E. coli R1, and the digested DNA has been fractionated by reverse phase chromatography on the resin, kPC5. DNA fractions from RPC5 chromatography have been subjected to electrophoresis on 1% agarose gels, transferred to nitrocellulose filters, and hybridized with either radioactive vitellogenin cDNA or mRNA.

Double-stranded cDNA has been prepared from purified vitellogenin mRNA and the DNA fragments have been introduced into the plasmid PBR322 using a "G-C" tailing procedure. Recombinant plasmids have been used to tranform E. coli X1776 under P3 physical containment conditions.

Major Findings: It has been determined from cDNA hybridization studies that the livers of normal roosters contain from 0-5 molecules of vitellogenin mRNA per cell. An increase in vitellogenin mRNA concentration was detectable within 30 minutes of administering either a primary or secondary injection of 17β-estradiol. The rate of accumulation of mRNA following a primary injection, however, was found to be biphasic. Vitellogenin mRNA accumulated at a rate of only 50 nucleotides $sec^{-1}.cell^{-1}$. for the first 4 hours following injection of the hormone and then the rate of accumulation increased during the next 8 hours to reach 350 nucleotides $sec^{-1}.cell^{-1}$. In contrast, a secondary injection of hormone administered to a completely withdrawn bird was found to elicit an initial rate of accumulation of 350 nucleotides $sec^{-1}.cell^{-1}$ which subsequently increased to approximately 540 nucleotides $sec^{-1}.cell^{-1}$. Following both a primary and secondary injection of hormone vitellogenin mRNA decayed with an apparent half-life of 30 hours. The increased vitellogenic response to secondary stimulation was found to be proportional to the increase in vitellogenin mRNA concentration. The results indicate that primary treatment with estrogen results in a long lived alteration either in the availability of the vitellogenin gene for transcription or in the persistence of a mechanism for stabilizing vitellogenin mRNA during the first few hours of the vitellogenic response.

Chicken DNA that had been digested with the restriction enzyme E. coli R1 yielded four fragments that hybridized with vitellogenin mRNA. Two of these fragments also hybridized to vitellogenin cDNA that was complementary to the 3' proximal 1000 nucleotides of vitellogenin mRNA. The aggregate size of the genomic DNA fragments that contain parts of the vitellogenin gene was found to be approximately 25,000 base pairs, indicating that the gene is most probably interrupted by sequences not represented in the mature mRNA. Vitellogenin dscDNA sequences have been cloned in the host/vector system λ1776/PBR322. Approximately 125 vitellogenin clones have been obtained that contain fragments of vitellogenin DNA ranging in size from 100 to 1000 nucleotides. Plasmid DNA from several of these clones has been prepared and attached either to nitrocellulose or diazotized paper filters for use as probes in in vitro transcription studies.

Significance to Cancer Research: It can be assumed that at least some forms of cancer can be attributed to aberrations in the mechanisms that control gene expression. At present, we have minimal knowledge of the processes that control the expression of genes at the transcriptional or the translational level in either the normal or malignant cell. One possible way to study these processes is to examine biochemically the ways in which certain agents can affect specific gene expression. An ideal system for these studies is one in which hormones that can regulate the synthesis of specific proteins are employed. In the studies described above, estrogen is used to induce the synthesis of vitellogenin in the rooster, an animal in which the vitellogenin gene is completely repressed in the normal condition. In addition, the synthesis of vitellogenin is appropriate for the study of controls of regulation of post-translational modification of proteins, since vitellogenin is a protein that is phosphorylated and glycosylated after or during its translation and it is presently not known how or to what extent post-translational modifications influence specific protein synthesis and transport.

Proposed Course of Research: Work will continue on the fractionation of the chicken genome with a view to cloning large fragments of DNA containing both the structural and regulatory elements of the vitellogenin gene. These DNA fragments will ultimately be used in a reconstituted in vitro transcription system utilizing homologous RNA polymerase, histones and nonhistone proteins. This system will be used to study the molecular mechanisms involved in regulation of expression of the vitellogenin gene.

Publications:

Goldberger, R.F., and Deeley, R.G.: Autogenous regulation of gene expression. In Marzluf, G.A. and Copeland, J.C. (Eds.), Regulatory Biology. Columbus, Ohio State University Press, 1977, pp. 178-195.

Deeley, R.G., Gordon, J.I., Burns, A.T.H., Mullinix, K.P., Bina-Stein, M., and Goldberger, R.F.: Primary activation of the vitellogenin gene in the rooster by estrogen. J. Biol. Chem. 252: 8310-8319, 1977.

Gordon, J.I., Deeley, R.G., Burns, A.T.H., Paterson, B.M., Christmann, J.L., and Goldberger, R.F.: In vitro translation of avian vitellogenin messenger RNA. J. Biol. Chem. 252: 8320-8327, 1977.

Deeley, R.G., Udell, D.S., Burns, A.T.H., Gordon, J.I., and Goldberger, R.F.: Kinetics of avian vitellogenin messenger RNA induction: Comparison between primary and secondary response to estrogen. J. Biol. Chem. 252: 7913-7915, 1977.

Burns, A.T.H., Deeley, R.G., Gordon, J.I., Udell, D.S., Mullinix, K.P., and Goldberger, R.F.: Primary induction of vitellogenin mRNA in the rooster by 17β-estradiol. Proc. Nat. Acad. Sci. USA 75: 1815-1819, 1978.

Deeley, R.G., and Goldberger, R.F.: Regulation of specific gene expression in rooster liver. In Hamilton, T.H., and Clark, J. (Eds.), Ontogeny of Receptors and Molecular Mechanisms of Reproductive Hormone Action, New York, Raven Press, 1978, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB05242-02 LB

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Organization of Estrogen-Responsive Genes in the Avian Genome

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	F. C. Eden	Staff Fellow	LB NCI
OTHER:	R. F. Goldberger	Medical Director (Chief)	LB NCI
	J. P. Hendrick	Chemist	LB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Cellular Regulation Section

INSTITUTE AND LOCATION

DCBD, NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.1	1.2	0.9

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The aim of this project is to determine the organization of structural genes in the avian liver whose transcription is altered by administration of estrogen.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: One of the most promising systems for the study of the regulation of gene expression is the production of the egg yolk protein vitellogenin in the avian liver in response to the hormone estrogen. The purpose of this project is to develop our knowledge of the structural organization of the avian genome to the point where specific questions can be asked about those particular regions of the genome that participate in the biosynthesis of vitellogenin and its regulation. Our ultimate aim is to identify other structural genes that also respond to estrogen, and to compare patterns of DNA sequence organization adjacent to a set of structural genes whose expression is co-ordinately controlled by estrogen administration.

Methods Employed: We have used two independent approaches to characterize the chicken genome and have also initiated an analysis of cloned fragments of the vitellogenin gene, which will be used as probes to characterize the structural features of the chicken genome that are adjacent to the vitellogenin gene.

1) We have now obtained a detailed description of the chicken genome and its sequence organization using hydroxyapatite chromatography. Both the quantity of single copy and repeated sequences and their arrangement relative to one another have been determined. We have also determined the predominant lengths of both single copy and repeated sequences using hydroxyapatite chromatography, SI nuclease digestion and gel filtration chromatography, and optical hyperchromicity of partially reassociated DNA fragments. These biochemical data have been confirmed and extended by electron microscopy of partially reassociated chicken DNA.

2) By hybridizing isolated single copy or repetitive fractions of chicken DNA to the DNA of other related avian species, we have been able to identify and characterize fractions of the chicken genome that have been highly conserved during avian evolution. Techniques have been worked out for the direct isolation of chicken genes homologous to the DNA of closely or distantly related birds, and we itend to investigate the relationship of these highly conserved sequences to the different patterns of sequence organization that occur within the chicken genome, and, eventually, to regions of the chicken genome that respond to estrogen.

3) Colleagues in the laboratory have succeeded in isolating recombinant DNA clones prepared using cDNA to highly purified mRNA. We are now participating in the characterization of these clones, using the methods of restriction analysis, hybridization to Southern blots using vitellogenin cDNA to determine the sizes of the inserted fragments, and mapping of the clones with respect to the 3'-OH terminus of vitellogenin mRNA by electron microscopy of R-looped structures. We are also developing the necessary technology (low temperature agarose gel electrophoresis followed by hydroxyapatite chromatography) for preparative isolation of the cloned, inserted fragments, with the aim of labeling these fragments in vitro for use as probes that will be absolutely specific for the vitellogenin gene.

Major Findings:

1) DNA Sequence Organization in the Chicken

Our present knowledge of DNA sequence organization in the chicken indicates that this genome represents a pattern intermediate between the extremes of DNA sequences organization known in other eukaryotes. About one half of the chicken genome consists of a regularly alternating pattern of single copy and repetitive sequences, with the average length of single copy sequences about 4500 nucleotides. The other half of the genome seems to consist almost entirely of long single copy regions uninterrupted by repeated sequence elements for very long distances. Thus, the chicken genome can, and has been, preparatively fractionated into interspersed and non-interspersed portions. By analysis of the reassociation kinetics of these isolated fractions with total DNA, we have obtained strong confirmation of the pattern of organization described above. This important result indicates that the chicken genome provides a unique opportunity to relate structure and function. This is so because we can locate specific genes (such as vitellogenin) within the interspersed or non-interspersed regions of the genome, and obtain direct evidence about the structural organization of DNA surrounding an estrogen-responsive gene. In addition, we have determined that the chicken genome contains an unusually small fraction of repeated DNA, and that these repeated sequences are much longer than the short (300 nucleotide) repeated sequences typical of most eukaryotic genomes. The implication of these two findings is that the number of different repeated sequences in the chicken genome may be much smaller than that in other genomes, and may provide a good opportunity to obtain a complete description of the distribution of repetitive families within the genome as a whole.

2) Identification of the Conserved Fraction of Chicken Repetitive DNA

We have applied a new combination of preparative steps aimed at isolating fractions of the chicken genome that have been evolutionarily conserved. These methods should find widespread application in the study of the evolution of DNA sequence organization, one of the most promising areas of study for the purpose of relating genome organization to regulatory functions. The major technical advance has been to enzymatically add a homopolymer to the ends of DNA fragments prior to hybridization with the DNA of another species. The DNA fragments that bear sequences homologous to the heterologous DNA can then be re-isolated after the hybridization using affinity chromatography specific for the homopolymer tract. Using this procedure, we obtained a fraction of chicken repetitive DNA that represents sequences conserved during at least 60 million years of avian evolution. By re-hybridization of this conserved fraction with chicken DNA and the DNA of other avian species, we were able to determine that although the sequences themselves have been conserved, their repetition frequencies in the respective genomes have undergone a hundred-fold change during the course of evolution. This study provides a new clue about the function of repetitive DNA: the nucleotide sequences themselves are probably more important than the number of copies of any particular sequence, since it is this feature that has been conserved evolutionarily.

It is our intention to use this conserved fraction of DNA, as well as individual cloned sequences representing it, to investigate the structural relationship between conserved repetitive sequences and expressed genes in general and the vitellogenin gene in particular.

3) Characterization of Vitellogenin Clones

Together with others in the laboratory, we have worked out the necessary methodology for isolation of recombinant plasmid DNA in workable amounts, restriction analysis of the clones, and identification of the inserted fragments by Southern blotting. We have, in one case, been able to confirm the size of the insert by direct observation of R-looped cloned DNA fragments in the electron microscope. We are in the process of gathering together a library of vitellogenin clones, with well-characterized inserts, which will then be used to perform mapping experiments to locate the cloned sequences with respect to the 3'-OH terminus of vitellogenin messenger RNA. Progress so far has included the isolation on a preparative scale of restriction fragments of vitellogenin clones, successful hybridization of vitellogenin mRNA to these DNA fragments with the formation of R-loops, and documentation of the size of the R-loops by electron microscopy. It remains to work out technology for extension of the very large vitellogenin mRNA molecule during spreading for electron microscopy, and preparation of a poly-A-specific "tag" for the poly A portion of the message.

We have also undertaken an exploratory series of experiments to determine the feasibility of secondary structure mapping of the vitellogenin message. Due to the large size of this RNA and its coding capacity for a very large polypeptide which subsequently undergoes a specific cleavage, it seems likely that this message may have evolved by fusion of previously separate genes at the level of DNA. Remnants of this fusion process may be visible in the present secondary structure of the message.

Progress so far in this area has been limited to establishment of conditions for partial denaturation of the message during spreading for electron microscopy. It remains to be seen whether a specific secondary structure feature can be identified and mapped.

Significance to Cancer Research: The basic mechanisms whereby differentiated cells undergo alterations in gene expression in response to extracellular stimuli are of direct relevance to understanding the establishment of malignant phenotype. We have chosen to study the structural features in DNA sequence organization that are involved in the alteration of cell phenotype in a hormone-responsive tissue. This information will apply directly in the search for the underlying structural features of animal genomes that are involved in differentiation in normal cells, and which undoubtedly are also related to transcriptional changes associated with malignancy.

Proposed Course of Research: We have prepared several new reagents for studying the regulation of gene expression in the chicken genome, and the following is a brief description of how each of these reagents will be used.

1) Cloned, labeled fragments of the vitellogenin gene will be used to determine whether the vitellogenin gene (or genes!) is located in the interspersed or non-interspersed fraction of the chicken genome. This same probe will be used to measure the rate of nucleotide substitution in that fragment of the gene during avian evolution. This rate can be related to that already measured for chicken single copy DNA as a whole. Subsequent derivative projects will include a comparison of the location of the vitellogenin gene in related avian genomes (as far as evolutionary divergence will allow sufficient hybridization). This project will answer the question whether the vitellogenin gene is always located in an interspersed arrangement of single copy and repeated sequences, or whether its location in varying avian genomes differs in this respect. We will also know whether vitellogenin is a conserved gene during avian evolution. This study will then be extended to compare cloned fragments that come from different regions (3" or 5' proximal) of the vitellogenin molecule. Since the vitellogenin message codes for at least two distinct polypeptides which may undergo different selection pressures in evolution, it would be of interest to determine whether all regions of the message are equally conserved, or whether some are drifting more rapidly than others. These data could eventually be compared to the corresponding protein sequence data when it becomes available.

2) We have obtained permission to clone an isolated repetitive fraction of chicken DNA. Clones will be screened with labeled probes representing the conserved repetitive fraction, kinase-labeled RNA from estrogen-induced liver, and kinased RNA from uninduced avian liver. These screens will allow selection of conserved repetitive sequences and sequences whose abundance in nuclear RNA changes in response to estrogen, respectively. We will use these clones, in turn, to determine whether the vitellogenin gene is adjacent to a conserved and/or an expressed repetitive sequence element in chicken DNA. As soon as an adjacent sequence is identified, it can be used to isolate other regions of the chicken genome that also contain members of that same repetitive family. The eventual end of this approach will be the isolation of a fraction of the chicken genome that is structurally related to sequences near the vitellogenin gene, and analysis of that fraction should provide a direct means of relating genome organization to function, i.e. responsiveness to estrogen.

Publication: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB05243-02 LB

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

In Vitro Synthesis of Vitellogenin Messenger RNA

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K. P. Mullinix	Research Chemist	LB NCI
OTHER:	J. L. Christmann	Staff Fellow	LB NCI
	J. D. Eldridge	Biologist	LB NCI
	M. B. Meyers	Research Chemist	LB NCI
	R. F. Goldberger	Medical Director (Chief)	LB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Cellular Regulation Section

INSTITUTE AND LOCATION

DCBD, NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	2	1

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have developed in vitro systems to use in the study of mechanisms by which estrogen influences specific gene expression. These systems include in vitro transcription in nuclei and chromatin from livers of normal and estrogen-treated roosters.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: In a previous study we showed that the administration of estrogen to roosters causes the de novo synthesis of vitellogenin messenger RNA in the liver. This mRNA is not synthesized by normal rooster. Thus, induction of the synthesis of vitellogenin mRNA by estrogen is an ideal model system in which to study the mechanisms by which the steroid hormone, estrogen, induces the expression of an otherwise inactive gene. We have attempted to characterize several in vitro systems that we will use to study expression of the vitellogenin gene. This work includes studies on transcription in isolated nuclei and in chromatin.

Methods Employed: We have developed a procedure for isolating nuclei that are active in transcription from rooster liver. This procedure involves the homogenization of liver in isotonic sucrose and the purification of the nuclei. We have also begun to study transcription in chromatin from normal and estrogen-stimulated roosters. Nuclei are isolated and chromatin is prepared by lysis of nuclei in hypotonic medium. The chromatin is not sheared prior to its use as a transcriptional complex and the chromatin-directed RNA synthesis that we have studied has utilized only the endogenous, chromatin-associated DNA-dependent RNA polymerase. We studied a variety of methods to monitor RNA that is synthesized in vitro. These have included labeling of in vitro synthesized RNA with mercurated nucleotide triphosphates and isolation of the mercurated RNA on sulfhydryl agarose columns, and labeling of in vitro synthesized RNA with ^{3}H-UTP or ^{32}P-UTP followed by isolation of the RNA and hybridization of this RNA to appropriate DNA sequences (See NCI ZO1 CB 5238-09 LB and NCI ZO1 5250-01 LB) immobilized on various paper supports.

Major Findings: Nuclei isolated by our procedures from rooster liver are pure as measured by phase contrast microscopy and are very active in transcription. The conditions used for RNA synthesis involve the use of relatively high levels of nucleotide triphosphates, and a triphosphate generating system (phosphocreatine and phosphocreatine kinase). The synthesis of RNA is linear for approximately 30 minutes. Both DNA dependent RNA polymerases I and II are active in our preparations, 62% of the synthesis being due to RNA polymerase II as measured by the sensitivity of this synthesis to α-amanitin. The nuclei incorporate approximately 4 picomoles of UMP into RNA per μg of nuclear DNA in 10 minutes of incubation at 25°C, a level of synthesis consistent with some reported in the literature for nuclei from other sources. We have found that nuclei from estrogentreated rooster liver are approximately twice as active in transcription as are those from livers of untreated roosters. We have had considerable difficulty in isolating high molecular weight RNA from our preparations, since the levels of ribonuclease in these nuclei are so high. We are still in the process of trying to overcome this problem. Chromatin isolated from rooster liver nuclei by our procedures is also active in transcription, utilizing the endogenous chromatin-associated RNA polymerases. The chromatin directs RNA synthesis to the extent of incorporation of 5 pmoles of UMP into RNA per μg of DNA in 10 minutes of incubation at 25°C. This synthesis is 50% inhibited by α-amanitin. An extensive series of experiments in

which we used Hg•UTP and Hg•CTP to label newly synthesized RNA has convinced us that this method is not suitable for obtaining an accurate estimate of the amount of newly synthesized vitellogenin mRNA in nuclei. We found that even under rigorously denaturing conditions it was not possible to remove all of the contaminating endogenous vitellogenin sequences from the newly synthesized RNA. We added an amount of vitellogenin RNA comparable to that found in nuclei isolated from estrogen stimulated rooster liver to the Hg•CTP labeled transcription products from control nuclei. We found that after the sulfhydryl sephadex chromatography under denaturing conditions, 25% of the added vitellogenin RNA sequences still remained. Thus we decided that the experiment to determine the amount of vitellogenin RNA sequences transcribed in vitro could only be done utilizing a cloned vitellogenin sequence and hybridizing ^{3}H-UTP or ^{32}P-UTP-labeled RNA synthesized in vitro to vitellogenin DNA sequence under conditions of vast DNA excess. Such a cloned vitellogenin DNA sequence has recently become available (See NCI Z01 CB 5238-09 LB). As model system, we have immobilized DNA from the plasmid pBR322 on either nitrocellulose filters or on diazotized M-aminobenzyloxymethyl cellulose paper (Alwine, J.C., Kamp, D.J., and Stark, G.R. (1977) Proc. Nat. Acad. Sci. USA 74: 5350-5354) and have hybridized ^{32}P-UMP labeled cRNA synthesized with E. coli RNA polymerase to the immobilized DNA. We have established conditions for hybridization and have done thermal melting profiles of the hybrids to prove that the binding of ^{32}P UMP labeled RNA that we observed was due to true hybrid formation. We are presently in the process of preparing large quantities of the plasmid pBR322•vitellogenin to immobilize on filters. As a control, we will test the ability of nuclei from normal and from estrogen-stimulated roosters to synthesize albumin RNA since albumin RNA synthesis should not be affected by estrogen. A plasmid containing the albumin DNA sequence has recently been prepared in this laboratory (See NCI Z01 CB 5250-01 LB).

Significance to Cancer Research: The elucidation of mechanisms that control biosynthetic processes is crucial to understanding cancer. Study of the means by which estrogen influences RNA synthesis will help determine how RNA synthesis is controlled. The study of vitellogenin mRNA synthesis in rooster liver has the great advantage that we can study the expression of a normally dormant gene in a differentiated tissue that has the capability of synthesizing RNA of a very large number of types.

Proposed Course of Research: We will establish the levels of transcription of vitellogenin and albumin RNA sequences in nuclei of normal and estrogen stimulated rooster liver. These determinations will also be done for chromatin. The chromatin from normal rooster liver will then be used as part of our in vitro system to attempt to transcribe the vitellogenin gene utilizing RNA polymerase and other nuclear proteins isolated from estrogen-stimulated rooster liver (See NCI Z01 CB 5247-01 LB).

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB05246-01 LB

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Organization and Expression of Genetic Information in Lower Eukaryotes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. A. Kramer	Staff Fellow	LB NCI
OTHER:	J. I. Smallwood	Chemist	LB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Cellular Regulation Section

INSTITUTE AND LOCATION

DCBD, NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project employs recombinant DNA techniques to isolate individual genes of yeast. These clones are then used in studies of the organization and expression of these genes at the molecular level.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: The goal of our research is to contribute to an understanding of the ways in which the genes of an organism are arranged and of the molecular mechanisms involved in the expression of genetic information. The yeast Saccharomyces cerevisiae was chosen as a model system for this project because extensive genetic studies have been carried out with yeast and because yeast has a relatively small genome. While yeast is a relatively simple eukaryotic organism, it has many molecular biological properties similar to those of higher organisms and not found in prokaryotes. Thus, yeast provides a relatively simple system for studying eukaryotic gene organization and expression at the molecular level.

Methods Employed: Recombinant DNA techniques are being used to clone individual yeast genes or groups of genes. The structure of these genes are then determined by restriction enzyme mapping, DNA sequence analysis and RNA mapping using hybridization and electron microscopy. Fully characterized clones are employed as probes to study in vivo gene expression and as templates for in vitro experiments to reproduce in vivo results. Transcription products are analyzed by RNA sequence analysis, gel electrophoresis, hybridization and RNA mapping techniques.

Major Findings: Because this project was initiated within the past year, much of the work has been preliminary and has not yet yielded significant results. A clone bank of bacteriophage λ carrying various EcoRI restriction fragments of yeast DNA was prepared and messenger RNA was extracted from yeast cells grown under varying conditions for use in differential screening of the clones. We have obtained some results with a cloned yeast gene isolated by the principal investigator prior to the initiation of this project at NIH, and these results will be described here.

Bacteriophage λ carrying the region coding for the ribosomal RNAs of yeast have been employed in a study of the expression of yeast genes in bacteria at the level of RNA synthesis. Hybridization assays show that yeast rDNA is transcribed in Escherichia coli, even in the absence of phage RNA synthesis. In addition, the transcription occurs on only one strand of the DNA, the same strand from which yeast rRNA is transcribed. Thus, the transcription of yeast rDNA in E. coli is initiated in the yeast DNA and shows some specificity. However, attempts to detect unique yeast transcripts in E. coli by preparative hybridization to cloned yeast rDNA followed by gel electrophoresis have been unsuccessful. Electron microscopic mapping of these transcripts does show RNA of significant size homologous to the yeast rDNA, implying that species of unique sizes necessary for detection on gels are not present due to non-specific transcription initiation or termination or to partial degradation of the transcripts. Quantitative analysis of the electron microscope data is underway to determine which possibility is most likely.

Significance to Cancer Research: An understanding of the molecular mechanisms involved in gene expression will be necessary to understand the processes by which a cell loses its genetic regulation and become cancerous. This project is designed to determine part of the normal mechanisms of gene expression and regulation as a basis for identifying the changes which have occurred at the molecular level in malignant cells. Knowledge of these changes should provide valuable information in the development of cancer prevention and treatment programs.

Proposed Course of Research:

1. Further studies of the expression of yeast rDNA in E. coli will be carried out in parallel with a detailed examination of rRNA synthesis in yeast. This will provide information about nucleic acid recognition sites for initiation and termination of transcription as well as RNA processing and about the conservation or divergence of these sites during evolution.

2. We are currently developing techniques which will facilitate the selection and identification of bacteriophage clones containing yeast genes which are induced or repressed under certain growth conditions. These clones will then be employed in the studies of gene organization and expression.

Publication: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05247-01 LB

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Isolation and Characterization of DNA-Independent RNA Polymerase II from Rooster Liver

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K. P. Mullinix	Research Chemist	LB NCI
OTHER:	W. H. Kastern	Guest Worker	LB NCI
	J. D. Eldridge	Biologist	LB NCI
	P. Leder	Medical Director (Chief)	LMG NICHD
	H. I. Miller	Research Associate	LMG NICHD

COOPERATING UNITS (if any)

Laboratory of Molecular Genetics NICHD

LAB/BRANCH

Laboratory of Biochemistry

SECTION

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INSTITUTE AND LOCATION

DCBD, NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1 3/4	1 1/4	1/2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have developed a procedure for isolating DNA-dependent RNA polymerase from rooster liver nuclei that involves treatment of nuclei with pancreatic DNAse I to partially solubilize chromatin. The enzyme thus obtained is completely dependent on the addition of exogenous DNA for activity. We have developed a technique for identifying RNA polymerase II in nuclear lysates by binding ^{3}H-α-amanitin to the polymerase and subsequently subjecting the proteins to electrophoresis in polyacrylamide gels under non-denaturing conditions. By this method we have identified two α-amanitin binding proteins, tentatively identified as two forms of RNA polymerase II.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: We plan to study the regulation by estrogen of the transcription of the vitellogenin gene in rooster liver. One possible approach to this problem is to reconstruct an in vitro transcription system, utilizing DNA dependent RNA polymerase isolated from normal or estrogen-treated rooster liver and chicken DNA in the form of "naked" total DNA, a cloned genomic vitellogenin sequence, chromatin, or various other templates. As a first step, we have sought to develop methods for isolating RNA polymerase that do not involve sonication of the chromatin since we think that sonication is a rather harsh treatment of a multisubunit enzyme. In addition, we have developed a method for identifying RNA polymerase in complex mixtures so that we can analyze the polypeptide composition of RNA polymerase without subjecting the enzyme to a rigorous purification procedure that might alter the composition of the enzyme.

Methods Employed:

Nuclei were isolated from rooster liver by homogenization of liver in isotonic sucrose followed by centrifugation on sucrose gradients. Nuclei were then lysed and subjected to digestion by DNAse I that we had coupled to Sepharose 4B. This procedure results in the partial solubilization of the chromatin. The digestion products are centrifuged and the supernatant fraction is collected and precipitated with $(NH_4)_2SO_4$. After dissolving the pellet, the enzyme is either used for transcription at this point or is further purified by chromatography on Sepharose 4B, DEAE-cellulose, DNA-cellulose and displacement chromatography (Torres and Peterson, in preparation). To localize RNA polymerase we have utilized the specific binding of α-amanitin to the enzyme. We obtained ^{3}H-γ-amanitin (gift of Dr. Roger Yocum, Biological Laboratories, Harvard University) and determined the conditions for binding to RNA polymerase. This stable amanitin-RNA polymerase complex was then subjected to electrophoresis on 2-16% or 4-30% gradient polyacrylamide gels (Pharmacia Fine Chem. Co.) under non-denaturing conditions. The gels were then sliced and the radioactivity in each slice determined. Finally, we have begun to assay the ability of RNA polymerase to transcribe various DNA templates in a specific way. These studies have included transcription of the chimeric plasmid PBR322-β globin 2 (done in collaboration with Drs. Henry Miller and Philip Leder) and SV40 Form 1 DNA.

Major Findings: DNA-dependent RNA polymerase II isolated as described above and precipitated with $(NH_4)_2SO_4$ is completely DNA dependent and transcribes both native and single-stranded calf thymus DNA. On single-stranded DNA the specific activity of the crude enzyme is 91.5 units/mg (1 unit = 1 pMole UMP incorporated in 30 min at 30°C). The α-amanitin binding experiments have shown that in rooster liver there are two α-amanitin binding proteins, having molecular weights of 640,000 and 580,000 and being present in a ratio of 2 (640,000) : 1 (580,000) at all stages of enzyme purification. The ^{3}H-α-amanitin bound to RNA polymerase is removed by competition with unlabeled α-amanitin. ^{3}H-α-amanitin does not bind to RNA polymerase isolated from E. coli.

The RNA polymerase was used to transcribe the chimeric pBR322-β globin 2 plasmid. The entire plasmid, both pBR322 and the β-globin 2 sequences, are transcribed and there was no evidence of specific transcription of the β-globin 2 sequence. We have just begun to study the extent of specific transcription of SV40 by this enzyme. We have utilized restriction endonuclease digestion of SV40 DNA by BamHI and HpaII to generate fragments of DNA corresponding to early and late mRNA coding regions. This digested DNA has been subjected to electrophoresis in agarose gels under denaturing conditions to separate strands. This material has been transferred to nitrocellulose filters by the Southern blotting technique and we can ask if the enzyme transcribes symmetrically or asymmetrically and if the enzyme starts at the in vivo origin of transcription.

Significance to Cancer Research: We are primarily interested in studying the control of gene expression, an aspect of regulation that is of primary importance in cancer, a disease in which regulatory processes become aberrant. By constructing an in vitro transcription system whereby we can study specific gene expression, we can elucidate some of the components necessary for correct regulation of transcription.

Proposed Course of Research: We have isolated RNA polymerase from rooster liver and have characterized the conditions necessary for in vitro activity. We have also developed the conditions for isolation of chromatin from rooster liver nuclei that is active in transcription (see NCI Z01 CB 5243-02 LB). We plan to analyze the transcription of vitellogenin sequences in chromatin from normal and estrogen stimulated rooster liver and examine the ability of RNA polymerase from normal and estrogen-stimulated roosters to transcribe the vitellogenin gene from chromatin. We also plan to continue studies of transcription of SV40 by this enzyme and to transcribe the genomic vitellogenin sequence when the sequence has been cloned.

Publications:

Kastern, W.H., Eldridge, I.D., and Mullinix, K.P.: Isolation and characterization of DNA-dependent RNA polymerase II from rooster liver. Abst. Annual Meeting of Soc. for Cell Biology, 1978, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB05248-01 LB

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

DNAse I Digestion of Nuclei from Chicken Liver and Chicken Erythrocytes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K. P. Mullinix	Research Chemist	LB NCI
OTHER:	M. B. Meyers	Research Chemist	LB NCI
	R. G. Deeley	Expert	LB NCI
	R. F. Goldberger	Medical Director (Chief)	LB NCI

COOPERATING UNITS (if any)

None

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SECTION

Cellular Regulation Section

INSTITUTE AND LOCATION

DCBD, NCI NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1 1/2	3/4	3/4

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have studied the susceptibility of the chicken globin gene in erythrocyte nuclei and the vitellogenin gene in liver nuclei to DNAse I pancreatic digestion. Whereas the globin gene was preferentially digested in erythrocyte nuclei, we were unable to show preferential digestion of the vitellogenin gene in liver nuclei isolated from roosters that were actively synthesizing vitellogenin mRNA.

HS-6040
(Rev. 10-76)

Project Description:

Objectives: It was shown by Weintraub and Groudine (Science 193: 848-856, 1976) that the globin gene of avian erythrocytes was preferentially digested by limited exposure to DNAse I. Even after the globin gene was not actively being transcribed, the susceptibility to DNAse I digestion persisted. These workers suggested that during the process of being transcribed genes assume a conformation in which they are more susceptible to DNAse I digestion than are non-transcribed genes. We sought to determine whether stimulation by estrogen would cause the vitellogenin gene in chicken liver nuclei to be preferentially digested by DNAse I. This experiment was designed to measure the susceptibility of the chicken vitellogenin gene to DNAse I digestion before, during, and after stimulation by estrogen. It is known that the vitellogenin gene is not transcribed in normal rooster liver and that vitellogenin mRNA synthesis can be induced by estrogen. After withdrawal from estrogen, vitellogenin mRNA synthesis ceases.

Methods Employed: Nuclei were prepared from chicken red blood cells and from chicken liver by a variety of procedures. The isolated nuclei were then incubated with pancreatic DNAse I under conditions in which 10-40% of the DNA was solubilized, depending on the experiment. The residual DNA was deproteinized and treated with ribonuclease. Some of the DNA was subjected to electrophoresis on polyacrylamide gels containing urea in order to monitor the extent of the DNAse I digestion. The remainder of the DNA was then analyzed by hybridization to various cDNA probes.

Major Findings:

1. Chicken erythrocyte nuclei.

Control nuclei and nuclei that had been digested with DNAse I were processed as described in Methods and the DNA was hybridized to chicken globin cDNA and to chicken vitellogenin cDNA. The DNA from control nuclei hybridized as expected to both globin and vitellogenin cDNAs. The DNA from nuclei that had been digested with DNAse I hybridized to vitellogenin cDNA exactly as the control DNA whereas the hybridization pattern with globin cDNA was altered. There was a depletion of the globin sequences from the digested DNA, indicating that the globin gene had been digested from erythrocyte nuclei to an extent that made it unable to hybridize with globin cDNA. These results are in accord with the results of Weintraub and Groudine.

2. Chickecn liver nuclei.

The experiment was conducted as described in Major Findings (1) in order to ask if the vitellogenin gene is selectively solubilized by DNAse I from nuclei actively synthesizing vitellogenin. We tested control and DNAse I-digested nuclei from estrogen stimulated roosters by hybridizing the nuclear DNA to vitellogenin cDNA. We found that there were no detectable differences in the extent of hybridization to vitellogenin cDNA between control nuclei and DNAse I-digested nuclei, indicating that by this method we could not observe

selective digestion of the vitellogenin gene by DNAse I under conditions in which we know that vitellogenin mRNA was being synthesized. We did a variety of experiments in which we varied the amount of DNAse I digestion and in which we altered the methods of nuclei preparation and repeated the hybridization experiment. We could not detect selective digestion of the vitellogenin gene by DNAse I under any of the experimental conditions that we tried.

Significance to Cancer Research: The mechanisms by which certain genes are selected for transcription are unknown. Understanding of these mechanisms is crucial to the understanding of the cancer process since the malignant cell clearly has lost essential mechanisms of control of transcription and gene expression.

Proposed Course of Research: One possible explanation for the apparent lack of selective digestion of the vitellogenin gene from nuclei of livers of roosters actively synthesizing vitellogenin mRNA is that only a fraction of the hepatocytes are active in vitellogenin synthesis. The remaining cells would have a quiescent vitellogenin gene, not susceptible to DNAse I digestion. We are currently examining a number of techniques to determine what proportion of the liver cells are active in vitellogenin synthesis.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB05249-01 LB

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

The Estrogen-Responsive Domain of the Rooster Liver

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. G. Deeley	Expert	LB NCI
OTHER:	C. R. King	Chemist	LB NCI
	D. S. Udell	Biological Aide	LB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

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SECTION

Cellular Regulation Section

INSTITUTE AND LOCATION

DCBD, NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	.2	.8

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The work described herein consists of an examination by cDNA complexity analysis of the changes that occur in the total mRNA population of rooster liver following treatment with 17β-estradiol. It has been demonstrated that the hormone markedly affects the concentration of no more than approximately 200 RNA species and that its major effect is the induction of a few very abundant species of mRNA. It has been possible to isolate a cDNA probe that is specific for these sequences and which will provide a suitable means of selecting clones containing estrogen-inducible sequences.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To analyze the complexity and concentration distribution of the mRNA population of rooster liver before and after treatment with 17β-estradiol and to determine the number of mRNA species that change in concentration following estrogen treatment, with the intention of isolating a complementary DNA probe that is specific for those sequences, for use in cloning studies.

Methods Employed: Total RNA has been extracted using guanidine•HCl from the livers of roosters before, during and after treatment with 17β-estradiol. Complementary DNA has been synthesized from the poly(A)-containing RNA present in these preparations with avian myeloblastosis virus reverse transcriptase. Such preparations of cDNA have been hybridized to their template RNA under conditions of RNA excess. The kinetics of these reactions have been analyzed to derive information concerning the number of different mRNA sequences in the total population and their relative concentrations. These analyses have been extended by fractionating the cDNA preparations to obtain cDNA probes specific for particular abundance classes of mRNA. This has been achieved by carrying out preparative scale RNA excess hybridization reactions to R_0t values that have been previously determined from analytical studies. Those cDNA sequences that were driven into hybrid at a particular R_0t value have been isolated by S1 nuclease digestion or, in those instances when the unreacted cDNA population has been required, by chromatography on hydroxyapatite. These fractionated cDNA probes have been hybridized, both to their template RNA and to RNA isolated from the liver in a different state of hormonal responsiveness. A cDNA probe derived from the abundant class RNA present in the liver at the height of the vitellogenic response has been hybridized to RNA from the liver of an untreated rooster so that only those sequences which have been induced at least 10-fold by estrogen would remain unhybridized and these sequences have been isolated by hydroxyapatite chromatography. The kinetics of hybridization of this "estrogen-inducible" cDNA probe to liver RNA of both estrogen-treated and normal roosters has been examined.

Major Findings: Homologous, RNA excess, cDNA complexity analyses have indicated that approximately 14,000 different species of mRNA are present in rooster liver both before and after treatment with 17β-estradiol. It has been determined from heterologous complexity analyses, between liver RNA from an estrogen-treated rooster and cDNA obtained from the liver RNA of a normal rooster, that essentially all those mRNA species present before treatment with the hormone can be found following hormonal stimulation, and that they remain in approximately the same proportions. Similar experiments using liver RNA from normal roosters and fractionated cDNA prepared from liver RNA obtained from estrogen-treated birds have demonstrated that treatment with estrogen appears to result in the extensive accumulation of a few mRNA species that can be found in the "abundant" and "intermediate" classes of mRNA. No significant changes have been found in the RNA population of the "complex" class which contains 95% of the different mRNA species of the liver. A cDNA probe has been prepared that is specific for the most abundant of the estrogen-inducible sequences. This probe has completely reacted with its template RNA by a R_0t value of 10 mol.s.l^{-1}, while it has only reacted to the extent of

10% with the liver RNA of a normal rooster by a R_ot value of 1000 $mol.s.l^{-1}$. Thus, a cDNA probe has been isolated for a group of mRNA species the concentration of which is increased approximately 100-fold following the administration of estrogen. By analyzing the kinetics with which this cDNA probe hybridizes to its template RNA it has been possible to estimate that it is derived from a group of mRNA species that has a total complexity of approximately 15,000 nucleotides.

Significance to Cancer Research:

It can be assumed that at least some forms of cancer can be attributed to aberration in the mechanisms that control gene expression. At the present we have little knowledge of the processes that control the expression of specific genes and essentially none, of the mechanisms by which the expression of groups of genes may be coordinated. In the studies described above estrogen is used to induce extensive accumulation of a group of mRNA species not normally found at high concentrations in rooster liver. The cDNA probe that has been isolated will allow the cloning of this estrogen responsive group of genes and thus provide a means of studying the regulation, both of the individual members and also of the group as a whole.

Proposed Course of Research: The mRNA sequences represented in the estrogen inducible cDNA probe will be identified by hybridization of this probe to preparations of liver total RNA that have been separated by electrophoresis on agarose gels. Permission has been obtained to clone the members of this estrogen inducible group of sequences (IMUA-16). The cDNA clones obtained will be used to purify individual mRNA species which will be used as radioactive probes to study the organization of this group of genes in the chicken genome.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB05250-01 LB

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Purification of Albumin mRNA and Cloning of Albumin dscDNA

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. I. Gordon	Surgeon	LB NCI
OTHER:	R. G. Deeley	Expert	LB NCI
	A. T. H. Burns	Visiting Fellow	LB NCI
	J. L. Christmann	Staff Fellow	LB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

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SECTION

Cellular Regulation Section

INSTITUTE AND LOCATION

DCBD, NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The work described herein consists of the development of a general procedure for cloning and identifying double-stranded DNA fragments transcribed from specific mRNA sequences, contained within a mixed population of mRNA species. The procedure combines techniques of RNA purification, complementary DNA complexity analysis and recent advances in recombinant DNA technology. Using this approach it has been possible to obtain several clones which have been shown to contain DNA sequences derived from the mRNA that specifies chicken serum preproalbumin.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To develop a generally applicable procedure for cloning specific DNA sequences obtained by transcription of a mixed population of mRNA species and to apply this procedure to the cloning of fragments of the chicken albumin gene.

Methods Employed:

Total RNA has been extracted from rooster liver using high concentrations of guanidine•HCl. The RNA has been translated in a wheat germ cell-free translation system and shown to program the synthesis of intact preproalbumin. Albumin mRNA has been partially purified from this preparation by affinity chromatography on Poly(U) Sephadex G-10, alternating with centrifugation through isokinetic sucrose gradients. The RNA preparation used for subsequent stages of this study consisted of approximately 10% by weight albumin mRNA as determined by cell-free translation, electrophoresis in agarose gels under denaturing conditions and by cDNA complexity analysis. Complexity analysis of the mRNA population revealed an abundant class that comprised approximately 30% of the total polyadenylated RNA present in the preparation. A cDNA probe specific for this class of RNA species, known to be composed predominantly of albumin mRNA was isolated by a preparative scale RNA excess hybridization to a predetermined R_0t value. This cDNA probe was used as a means of preliminary identification of clones containing putative albumin DNA sequences. A procedure has been developed for the efficient synthesis of double-stranded DNA from polyadenylated RNA using avian myeloblastosis virus reverse transcriptase. The procedure involves the use of a rat liver cytosol ribonuclease inhibitor and results in yields of DNA three-fold greater than those obtained using standard procedures. The overall efficiency of the process of transcribing mRNA into dsDNA is approximately 50%. Double-stranded DNA has been synthesized from the partially purified albumin mRNA preparation and inserted into the plasmid PBR322, using a homodeoxypolymer-tailing procedure. Such chimeric plasmids have been used to transform the approved EK2 vector X1776 under P3 physical containment conditions. The entire population of clones has been screened with a cDNA probe specific for the most aberrant species of mRNA. Thirty clones have been identified. The first of the clones to be examined has been demonstrated to contain an albumin DNA sequence by the ability of plasmid DNA isolated from the clone to specifically block the translation of albumin mRNA in a wheat germ cell-free translation system.

Major Findings: Albumin mRNA has been shown by cell-free translation to represent approximately 15% by weight of the mRNA of rooster liver. During acute treatment with 17β-estradiol the relative concentration of albumin mRNA declines until it comprises approximately 8% of the liver mRNA population. This decline in relative concentration does not reflect a true decrease in the albumin mRNA content of the liver but rather is a result of dilution by other abundant mRNA species, such as vitellogenin mRNA, the synthesis of which is markedly dependent upon induction by estrogen. These results suggest that the regulation of albumin mRNA synthesis and degradation is not appreciably affected by the administration of estrogenic steroid.

Albumin mRNA has been purified approximately 33-fold from liver total RNA. Double-stranded DNA has been synthesized from such an RNA preparation and cloned in the EK2 host/vector system λ1776/PBR322. The efficiency of the cloning procedure developed is such that one clone has been obtained for every 4 ng of RNA transcribed. Thirty clones containing putative albumin sequences have been isolated, by screening with a radioactive cDNA probe that has been highly enriched for sequences complementary to albumin mRNA. The identity of one of these clones has been confirmed by hybrid arrested cell-free translation. This clone has been found to contain an albumin DNA sequence approximately 500 nucleotides long. Plasmid DNA isolated from this clone has been used to identify albumin mRNA present in preparations of liver total RNA following separation by electrophoresis on methylmercury-agarose gels. It has been demonstrated that albumin mRNA is approximately 2600 nucleotides long and that it specifies a form of preproalbumin that has a molecular weight of 72,000. The RNA has been shown to be approximately 600 nucleotides longer than the minimum length necessary to specify preproalbumin.

Significance to Cancer Research: It can be assumed that at least some forms of cancer can be attributed to aberrations in the mechanisms that control gene expression. At present we have minimal knowledge of the processes that control the expression of genes at the transcriptional or the translational level in either the normal or cancerous cell. One possible way to study these processes is to examine biochemically the ways in which certain agents can affect specific gene expression. A possible system for these studies is the induction of albumin synthesis in cells of nonhepatic origin by the formation of somatic cell hybrids with various hepatomas. In the studies described above DNA fragments of the albumin gene have been isolated that may be used as specific probes in such systems. Albumin DNA probes may also be used to monitor the persistence of a specific differentiated cell function during transformation.

Proposed Course of Research: The cloned fragments of albumin DNA will provide a probe for *in vitro* transcription systems which will be used to investigate the specificity of action of steroid hormones *in vitro*. They will also be used as a means of purifying albumin mRNA for use as a probe to investigate the organization of the albumin gene in the chicken genome. Fragments of genomic DNA containing albumin gene sequences plus flanking sequences will be cloned in an approved bacteriophage vector. Such clones will be used either as probes or substrates for *in vitro* transcription systems using partially purified preparations of chicken RNA polymerase II.

Publications:

Gordon, J.I., Burns, A.T.H., Christmann, J.L., and Deeley, R.G.: Cloning of a double-stranded cDNA that codes for a portion of chicken preproalbumin: A general method for isolating a specific DNA sequence from partially purified mRNA. *J. Biol. Chem.*, 1978, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB05231-04 LB

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Role of Subunit Interactions in Enzyme Chemistry

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C. B. Klee	Research Chemist	LB NCI
Others:	P. G. Richman	Staff Fellow	LB NCI
	T. H. Crouch	Staff Fellow	LB NCI

COOPERATING UNITS (if any) Dr. R. Adelstein, Dr. D. Hathaway, Cardiology Branch, NHLBI
Dr. P. Davies, LMB, NCI
Dr. L. Kohn and Dr. Magnuson, LBP, NIAMDD

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Nucleic Acid Enzymology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.4	3.0	2.4

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies on the mechanism of action of M. Luteus polynucleotide phosphorylase have shown that "de novo" synthesis catalyzed by the enzyme yields long polymers of defined chain length.

The homogenous Ca^{2+}-dependent cyclic nucleotide phosphodiesterase activated by the Ca^{2+}-dependent regulator (CDR), was shown to be the "high Km" enzyme which hydrolyzes both cAMP and cGMP. The enzyme has a molecular weight of 135,000±10,000, its catalytic subunit has a molecular weight of 59,000. Using chemically modified CDR, we were able to show that the catalytic subunit binds to the regulatory protein. It was also shown that tne enzyme interacts physically with the inhibitory protein previously described in this laboratory.

Other proteins under CDR control, smooth muscle and non-muscle cell myosin light chain kinases were purified by affinity chromatography on CDR coupled to Sepharose. Their regulation by CDR is presently under study.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To study the functional role of subunits and protein-protein interactions. Two enzymatic systems: polynucleotide phosphorylase and cAMP phosphodiesterase and other calcium dependent regulator protein systems are being studied.

Methods Employed: The project involves the study of properties of proteins, purified by the usual techniques of protein isolation, including many types of chromatography. The enzymes are being studied by examining their optical, hydrodynamic, and kinetic properties as well as by suitable chemical measurements and modifications.

Polynucleotide Phosphorylase.

Mechanism of action. During de novo polymerization of ADP or UDP the enzyme was shown to synthetize long chains of defined chain length prior to release the formed product. The exact size of the polymer determined by electron microscopy and the mechanism by which the enzyme can determine the proper number of nucleotide residues to be incorparated is still under study (E. Barbehenn and C. Klee).

Regulation of cAMP Phosphodiesterase.

Regulation of cyclic nucleotide phosphodiesterase by its Ca^{2+}-dependent regulator (CDR) is being studied as a model system for Ca^{2+}regulation mediated by CDR which is now recognized as multifunctional modulator of many enzymes. The Ca^{2+}binding properties of CDR have been analyzed at three levels: Equilibrium dialysis confirmed the existence of four Ca^{2+} binding sites, three of them having similar affinity constants (K_D= 6.4 x 10^{-6} M). Binding of the first Ca^{2+} appears to induce a conformational change different from that previously observed upon binding of two Ca^{2+} per mole of CDR. This structural change has been followed by near UV circular dichroism and ultracentrifugation. Finally it was shown that activation of phosphodiesterase by Ca^{2+} depends on the concentration of free CDR and that at least three Ca^{2+} bound per mole of CDR are required to activate CDR in this system (T. Crouch and C. Klee).

Selective chemical modification of the two tyrosine residues of CDR along with spectrophotometric titration studies of the unmodified and modified (nitrated) residues has helped to elucidate the separate microenvironments of these residues. The results indicate that tyrosine 99 is exposed to solvent and its microenvironment is not perturbed by Ca^{2+} binding to the protein. Tyrosine 138, in contrast, is in a unique microenvironment that is substantially altered by Ca^{2+} binding to CDR. Nitration of neither of the tyrosine residues affected the ability of CDR to activate phosphodiesterase. CDR was also labeled by [^{125}I] to the extent of one mole of monoiodotyrosine per mole or by guanidination of the lysine residues (one

mole of homoarginine per mole) without change in activity. The labeled CDR was used to demonstrate the formation of Ca^{2+}-dependent complexes of CDR with phosphodiesterase and its inhibitory protein (P. Richman and C. Klee).

The highly purified Ca^{2+}-dependent phosphodiesterase has been characterized. The enzyme has a molecular weight of 135,000±10,000. Gel electrophoresis under denaturing conditions revealed the presence of three polypeptides with apparent molecular weights of 61,000, 59,000 and 15,000. The catalytic activity for both cAMP and cGMP as well as the ability to be activated by CDR is associated with the 59,000 MW subunit. The other two polypeptides are the components of the inhibitory protein which also interacts with the enzyme. The physiological role of this protein is still to be determined. The kinetic behavior of the purified enzyme towards cAMP has been analyzed in detail. In the presence of CDR the enzyme obeys Michaelis-Menten kinetics, has a Km value of 100 µM for cAMP and 10 µM for cGMP. In the absence of CDR the enzyme exhibits negatively cooperative kinetics, and a very low Vm which is increased 10-fold by addition of CDR. The apparent Km of the enzyme for CDR is dependent on cAMP levels (1.5×10^{-9} and 3×10^{-9} M at 10^{-3} and 10^{-6} M cAMP respectively). Both imidazole and NH increase the Vm and Km values in the presence or absence of CDR. Imidazole greatly lowers the affinity of the enzyme for CDR as well.

In collaboration with Dr. Adelstein (NHI) we have purified myosin light chain kinases from smooth muscle and non-muscle cells by affinity chromatography. The activation of these proteins by CDR is presently being studied (R. Adelstein, D. Hathaway and C. Klee). Another class of proteins, which bind CDR, and appear to be cytosketal protein components is being investigated in callaboration with Dr. P. Davies (NCI).

Other collaborative projects include the study of the role of CDR and troponin C in the binding of TSH to thyroid membranes (L. Kohn, R. Magnuson and C. Klee).

Significance to Biomedical Research and the Program of the Institute:

Both proteins being studied are examples of regulation of catalytic activity by protein-protein interactions. Polynucleotide phosphorylase, a well-known bacterial enzyme, also has been described in mammalian cells. It is widely utilized for the synthesis of model nucleic acids used in nucleic acid enzymology and interferon induction, and also in the degradation of poly A sequences of mammalian mRNA. Its physiological role is still undetermined, but it would seem likely that the enzyme has an important function in nucleic acid metabolism.

cAMP phosphodiesterase, as one of the enzymes responsible for the control of cAMP levels which are critical for the regulation of cell growth and differentiation, is of considerable interest. The potential role of CDR in mediating the cellular effects of Ca^{2+} and the functional state of contractile proteins is of obvious importance. This ubiquitous regulatory,

protein could provide a link between cyclic nucleotide levels and multiple cell functions. The ability of CDR to regulate a large number of biological functions represents a novel mechanism with potential physiological significance.

Proposed Course of Research:

Studies on polynucleotide phosphorylase will be directed towards the elucidation of the mechanism of action of the enzyme: particularly its ability to synthetize polymers of precise chain length in the absence of a template. The role of tightly bound adenylate kinase and ATPase activities in the initiation of polymerization will be also investigated further.

cAMP phosphodiesterase activity is regulated by two naturally occuring proteins: Ca^{2+} dependent regulator and a CDR binding protein as described above. The effects of other types of positive or negative effectors such as imidazole and theophylline on the regulation of the enzyme by its physiological effectors will be studied. All studies will be conducted on enzyme or regulatory proteins purified to homogeneity in order to simplify the interpretation of the results. Subunits and protein-protein interactions will be studied in order to elucidate their potential role in the complex cooperative kinetics exhibited by the enzyme and the concerted regulation mechanism of CDR. Similar studies will be applied to the recently purified light chain myosin kinases in order to understand the multiple function of CDR. Subcellular localization of the various components will be studied with the aid of antibodies which also may be useful in studies of the regulation of synthesis and degradation of these proteins.

Publications:

Klee, C.B., and Krinks, M.H.: Purification of 3',5' nucleotide phosphodiesterase inhibitory protein by affinity chromatography on activator protein coupled to sepharose. Biochemistry, 17: 120-126, 1978.

Barbehenn, E.K., and Klee, C.B.: The origin of the electrophoretic microheterogeneity in primer-independent polynucleotide phosphorylase from micrococcus luteus. J. Biol. Chem. 253: 2446-2454, 1978.

Richman, P.G., and Klee, C.B.: Conformation dependent nitration of the protein activator of 3',5'-cyclic adenosine monophosphate phosphodiesterase. Biochemistry, 17: 928-935, 1978.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB05232-03 LB

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Studies on the Replication of Simian Virus 40 (SV40) DNA in Monkey Kidney Cells.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. C. Kay	Visiting Fellow	LB NCI
Other:	M. F. Singer	Head, Nucleic Acid Enzymology Section	LB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Nucleic Acid Enzymology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.4	1.2	0.2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Terminated

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB05234-04 LB

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Replication and Properties of Defective - Substituted Simian Virus 40 in Permissive Monkey Kidney Cells.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. F. Singer	Head, Nucleic Acid Enzymology Section	LB NCI
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NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.2	4.8	0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Nucleotide sequences have been determined in two defective variants of SV40 that contain covalently linked monkey and viral DNA sequences. In each variant, part of the monkey sequences are derived from the highly reiterated portion of the cellular genome called α-component. However each variant contains a different member of the set of closely related, 172 base pair long monomer units contained in α-component. Each variant also contains sequences tentatively identified as low reiteration frequency monkey sequences: the sequences in the two variants are different. Sequences around the joints between the SV40 and monkey DNA have been determined. These studies afford information on the nature of the recombinational events involved in the evolution of the variants.

Project Description:

Objectives: The discovery of variants of simian virus 40 that are substituted with DNA segments derived from the host cells, African Green monkey kidney cells, offers the opportunity to study recombination in a primate system. It is the purpose of this work to exploit these variant viral genomes to study the mechanism of recombinational processes, as well as aspects of the interaction of virus and host.

Methods Employed: Standard tissue culture procedures are used for the growth of monkey kidney cells and SV40. Procedures used for studying viral DNA synthesis included radioisotope tracer techniques, preparative and analytical ultracentrifugation, DNA-DNA hybridization, selective precipitation of high molecular weight DNA. Electron microscope is used to study the size and configuration of DNA and is also used to study DNA structure by heteroduplex analysis. Extensive use is made of restriction endonucleases and both preparative and analytical gel electrophoresis for the preparation of pure DNA fragments of defined size. A variety of methods are used for the determination of the sequence of nucleotides in segments of DNA. These include 1) preparing a cRNA copy of the DNA segment with E. coli RNA polymerase, analysis of the RNA sequence by complete and partial digestion with pancreatic and T1 ribonucleases and determination of the structure of the oligoribonucleotides produced and 2) direct DNA sequencing techniques using both partial snake venom phosphodiesterase digestion of terminally labeled fragments and the chemical procedures (methylation and hydrazinolysis) introduced by Maxam and Gilbert, also carried out on terminally labeled DNA fragments.

Major Findings:

Last year we described our progress towards understanding the structure of one cloned defective variant of SV40 (termed CVP8/1/P2) that contains both host and viral sequences in covalent linkage. We have now extended that study so that almost the entire nucleotide sequence of the 1200 base pair repeat unit of the defective genome is known. This has allowed elucidation of the nature of the host sequences not previously characterized. Most importantly, the sequences at all the joints representing recombinations between host and viral DNA, as well as an unexpected joint derived from deletion and inversion of certain viral sequences within the defective, are now known. In addition, the sequence of a second cloned recombinant defective SV40 genome has been studied. This second variant, 1103, was derived completely independently from the first, indeed, was obtained from stocks of a different wild type strain of SV40. In the case of 1103, as in the first instance with variant CVP8/1/P2, the monkey sequences in the variant have been almost completely determined (440 base pair repeat), as have all the joints between monkey and viral sequences. Thus, the two variants can be compared and we can begin to define the characteristics of such recombinants.

Interpretation of the data is facilitated because the entire nucleotide sequence of the 5226 base pairs of the wild type SV40 genome is now known from the work of others. In addition, the sequence of a 172 base pair segment that is repeated over a million times in the monkey genome (the so-called α-component), and occurs in both defective genomes, was determined in this laboratory (see Project Number: Z01 CB 5244-01 LB).

The two wild type parents of the defectives. Because the two defective variants under study were each derived from a different wild type parent, it was important to understand the differences, if any, between the two wild type strains. Therefore, the DNAs of the two strains, termed 777 and 776 were compared by using restriction endonucleases (it is the sequence of 776 which has been published). Differences between the two strains were detected at five separate points on the SV40 genome. One of these differences, in the region of DNA coding for the major viral coat protein, VP1, was confirmed by tryptic peptide analysis of coat proteins isolated from the two strains. The region coding for the coat protein is missing in each of the two defectives. However, each of the defective genomes is normally encapsidated in the coat protein of its parent, which serves as obligatory helper for replication of the defective. Thus, CVP8/1/P2 is encapsidated in a 777 coat, and 1103 in a 776 coat. As with many defective animal viruses, these two variants interfere with the replication of wild type virus. We have observed a specificity in this interference: CVP8/1/P2 interferes more efficiently with the replication of its parent, 777, than with the replication of strain 776. Preliminary experiments have indicated that there is no specificity for interference when infection by DNA itself is studied. Thus, it appears that the specificity resides in the capsids.

Wild type SV40 sequences in the variants. Both variants contain only short regions of wild type SV40 sequences and these include sequences within the region associated with the origin of replication. Thus the structure of these two variants is consistent with the view that the origin is the only "cis" requirement for replication. Variant 1103 has 155 bp of wild type sequence (region 0.65 to 0.68 on the wild type map) and variant CVP8/1/P2 contains about 425 base pairs of wild type sequence (region 0.62 to 0.70 and a short segment from map position 0.73 of the wild type). Thus, in the formation of CVP8/1/P2 two noncontiguous wild type regions were made contiguous in the defective. However, the short segment from about wild type map position 0.73 is inverted, in the defective, relative to the segment at the origin of replication (map position about 0.67).

Highly reiterated monkey DNA (α-component) in the defective variants. Both CVP8/1/P2 and 1103 contain sequences derived from α-component DNA. CVP8/1/P2 contains 157 of the 172 residues of the repeat unit of α-component. In two position transitions, compared to the sequence determined for the α-component monomer, have occurred.

Variant 1103 contains 143 of the 172 residues of the repeat unit of α-component. It differs from the determined α-component sequence in six positions, all different from the two differences in CVP8/1/P2.

The data thus suggest that each of the two variants contains a different member of the set of sequences comprising the α-component repeating unit. Furthermore, different, but overlapping regions of the α-component sequence appear in the two defectives. It is striking that the beginning and end of the α-component sequences in each of the two defectives are close to one another. A total of 8 of the 172 base pairs of α-component do not appear in either defective. This suggests some specificity in the recombinational events. Inverted repeat sequences, capable of forming stem and loop structures, occur relatively close to the beginning and end of the α-component segment within the two defectives.

Less frequently reiterated (or unique) monkey sequences in the defective variants. CVP8/1/P2 contains about 600 base pairs of sequence that is neither SV40 nor α-component. One portion of this has been identified, by hybridization kinetic studies, as monkey DNA of reiteration frequency 20 or less. Variant 1103 contains about 140 such base pairs, not yet identified positively as being of monkey origin, but assumed to be "unique". The sequences in 1103 are not homologous to those in CVP8/1/P2.

Both variants then contain contiguous stretches of monkey DNA much longer than a single α-component repeat unit. But, as pointed out above, each variant contains less than a single complete repeat unit of the α-component monomer even though the bulk of α-component sequences occur in long tandem repeats within the monkey genome. There are several possible explanations for this finding. First, if the sequences in the variants reflect the initial recombination events between virus and host, then rare copies of the α-component sequence were involved in the recombination. In the monkey genome these rare copies might be found at the ends of tandem repeats where α-component sequences join other DNA or else might represent an α-component segment that is interspersed between other DNA sequences. Since each of the defectives contains a different member of the set of α-component sequences, and different flanking low reiteration frequency sequences as well, a different such rare segment would have been involved in the formation of each of the two defectives. A second possible explanation is that the structures of the purified defectives represent multiple recombinational events and therefore do not indicate anything about the relation of the α-component sequences to the flanking sequences within the monkey genome. In the case of CVP8/1/P2 there is some evidence to suggest that the sequences closely flanking the α-component segment have not been markedly altered in the course of multiple amplifying passages. Thus, in the very first high multiplicity passage of plaque purified strain 777 in the passage series leading to CVP8/1/P2, a restriction fragment containing highly reiterated monkey sequences and corresponding in size to the analogous fragment obtained from CVP8/1/P2 was generated by endoR•HindII/HindIII. Although it has been suggested that defective variants of SV40 containing most sequences originate late in a series of high multiplicity passages, the history of CVP8/1/P2 suggests that they may also arise very early in course of the passaging.

Joint between SV40 and monkey DNA sequences in the defective variants. In CVP8/1/P2 there are two places where SV40 sequences are joined to monkey low reiteration frequency sequences. In this case, the only known sequences lost through the recombination are the SV40 sequences. In variant 1103 there is one place where SV40 sequences are joined to α-component sequence and one place where SV40 sequences are joined to low reiteration frequency monkey sequence. In the former joint, both sequences lost through the recombination are known.

Inspection of the sequences around the joints has led, thus, far to only limited conclusions. It is evident that there are no substantial regions of homology between the monkey sequences and the SV40 sequences, indicating that nonhomologous recombination mechanisms have operated during formation of the defectives. Regions with a relatively high content of AT base pairs seem to be common close to, but not necessarily right at, the joint. At this time, no feature of the sequences around the joints is suggestive of any particular recombination mechanism, but further study of these sequences is required.

Proposed Course of the Research:

1. Further study of the sequences already obtained will be made in order to look for features that might be indicative of a recombination mechanism.

2. We have already initiated studies designed to facilitate the detection and cloning of additional defectives. We are especially interested in methods for early detection, so that variants representing primary recombinational events can be isolated.

3. We would like to know if these recombinant variants arise because of integration of wild type virus into the host genome, during lytic infection. We will try to isolate monkey cells with integrated viral genomes and then study the host sequences contiguous with the viral sequences for their relation to those sequences that are known to occur in defectives.

4. We are planning additional physiological studies designed to elucidate further the mechanisms of interference by defective viruses on the replication of the wild type.

Publications:

Kay, A.C., Rao, K.G.R., and Singer, M.F.: Comparison of two viable variants of simian virus 40. J. Virol. 25: 339-348, 1978.

Singer, M.F., Rosenberg, M., Rosenberg, H., McCutchan, T., Wakamiya, T., and Segal, S.: Monkey DNA sequences in defective simian virus 40 variants. In, Persistent Viruses, ICN-UCLA Symposium, in press, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB05237-04 LB

PERIOD COVERED
October 1, 1977 to September 30, 1978.

TITLE OF PROJECT (80 characters or less)

Interaction of Histones with Superhelical DNA.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. F. Singer	Head, Nucleic Acid Enzymology Section	LB NCI
Other:	A. C. Kay	Visiting Fellow	LB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Nucleic Acid Enzymology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.7	0.1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The synthesis of histones during lytic infection of BSC-1 (African Green Monkey kidney) cells with Simian Virus 40 (SV40) has been investigated. The synthesis of all five classes of histones was stimulated, and all classes appeared to be stimulated to the same extent. The increase in rate of histone synthesis in response to SV40 infection was detectable several hours before SV40 DNA synthesis was measurable, and the rate of histone synthesis decreased at a time when SV40 DNA synthesis was occurring at a maximal or relatively high rate. In addition, the changes in rates of histone synthesis did not correlate well with the rates of host DNA synthesis during infection. Thus it appears that DNA synthesis and histone synthesis may not be strictly coupled in SV40 infected cells.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To study the interaction between mammalian histones and superhelical DNA and relate the interaction to the functional role of histones in DNA structure and metabolism. Emphasis is placed on SV40 DNA as a model for superhelical DNA.

Methods Employed:

Standard tissue culture techniques for growth of monkey kidney cells and for preparation of simian virus 40 (SV40) DNA. Cell fractionation methods for isolation of intracellular nucleoprotein complexes involving SV40 DNA. Subcellular fractionation, protein purification, radioisotope tracer techniques, gel electrophoresis, ultracentrifugation, nitrocellulose filter binding techniques, protein chemistry and amino acid analysis; enzymic techniques (involving polynucleotide ligase and "unwinding enzyme"), coupled with agarose gel electrophoresis for isolation and analysis of closed-circular duplex DNAs of known superhelicity; electron microscopic visualization of superhelical DNA and bound histones. In addition to radioautography, fluorography has been used to visualize radioactive material on polyacrylamide gels.

Major Findings:

In uninfected, growing cells, the synthesis of histones and of messenger RNA for histones is tightly coupled to the synthesis of cellular DNA. In order to investigate the relation between histone and DNA synthesis in a simpler, more defined system, we have studied the synthesis of histones that occurs upon infection of African green monkey kidney cells with SV40 and the consequent synthesis of viral DNA. One complication in such studies is that cellular DNA synthesis itself is stimulated upon viral infection. In order to minimize the contribution of cellular DNA synthesis, we used a line of monkey kidney cells in which the induction of cellular DNA synthesis is known to be less marked than usual.

It is known that viral DNA, inside the cell, and prior to packaging into virion particles, is associated with all five of the common histones, H3, H4, H2a, H2b, and H1, in a nucleoprotein particle called a minichromosome. Further, it is known that these histones are encoded by the host genome, not the viral genome. Thus, the stimulation of the synthesis of histones in infected cells represents a "turning on" of a host function.

We measured both the rate of synthesis of histones and the rate of synthesis of viral and host DNA at various times after infection. Rates of synthesis were measured by pulse labeling cells with either amino acid precursors of the histones, or pyrimidine precursors of DNA and isolation of the appropriate material. We demonstrated that infection of the monkey kidney cells with SV40 causes an increased rate of histone synthesis. Interestingly, this histone synthesis begins and declines several hours

before similar events are detected in the rate of SV40 DNA synthesis. Thus, for example, at 48 hours after infection, SV40 DNA synthesis is proceeding at a relatively high rate, but the rate of synthesis of histones is markedly depressed. Early in infection interpretation of the data are more complex, because cellular DNA synthesis is stimulated by 14 hours after infection, as is histone synthesis, although viral DNA synthesis is barely detectable at this point. Nevertheless, since cellular DNA synthesis continues at about the same level even late in infection, when histone synthesis has dropped sharply, it appears that there is no good correlation between cellular DNA synthesis and histone synthesis in these infected cells.

The overall conclusion from these experiments is that histone synthesis in SV40 infected cells is not tightly coupled to SV40 DNA synthesis.

There are several possible reasons for such a dissociation of histone synthesis from DNA synthesis. Late in infection the synthesis of large amounts of viral capsid proteins may require most of the protein synthetic apparatus of the cell, resulting in a decrease of histone synthesis before viral DNA synthesis is completed. The early start in histone synthesis may result in larger than normal histone pools which can be used for the SV40 DNA synthesized after histone synthesis is almost completely shut down late in infection. Another possibility is that infecting viral DNA, which lacks H1 (H1 is not detected in virions), needs H1 before it can begin replication. If the genes for all five histones are expressed coordinately, then synthesis of all histones may be turned on even if only H1 is needed. Later in infection, as the viral DNA and four smaller histones are encapsidated, H1 may accumulate in the cell and turn off histone synthesis. That histones may regulate their own synthesis has been proposed previously.

Synthesis of all five classes of histones are stimulated to about the same extent by SV40 infection. H1 is not detected in the completed virion but H1 can be found in association with the SV40 minichromosome within the infected cell. We carried out some preliminary studies on the metabolism of H1 during SV40 lytic infection and on whether the H1 synthesized in response to SV40 infection represents any particular one of the H1 subfractions. Using Amberlite-IRC50 columns in guanidine chloride, we separated H1 histone isolated from infected and uninfected cells into several of its subfractions. We obtained preliminary evidence that one particular subfraction of H1 is synthesized more actively than the others during rapid viral DNA synthesis in infected cells. We do not know if this represents an H1 fraction that is associated with DNA synthesis in general or with the synthesis of viral DNA in particular.

Significance to Cancer Research: The role of histones in 1) the structure of chromatin and 2) the regulation of gene expression and DNA replication in eukaryotic cells is under intensive investigation in many laboratories. The relevance of the control of eukaryote gene expression to the nature of oncogenic processes has been widely discussed.

Proposed Course of Research:

Terminate.

Publications:

Kay, A.C., and Singer, M.F.: Histone synthesis during infection of monkey kidney cells with Simian Virus 40. Nucleic Acids Res. 4: 3371-3386, 1977.

Singer, D.S., and Singer, M.F.: Characterization of complexes of supernelical and relaxed closed circular DNA with H1 and phosphorylated H1 histones. Biochemistry 17: 2086-2095, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB05244-01 LB

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Organization of Repeated DNA Sequences in African Green Monkeys.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. F. Singer	Head, Nucleic Acid Enzymology Section	LB NCI
Other:	D. S. Singer	Staff Fellow	LB NCI
	T. F. MuCutchan	Staff Fellow	LB NCI
	R. Thayer	Chemist	LB NCI

COOPERATING UNITS (if any)
Martin Rosenberg, Ph.D. LMB NCI

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.1	2.0	0.1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The genome of the African Green monkey (Cercopithecus aethiops) contains a class of highly repeated DNA. Between 10 and 20 percent of the total genome is made up of repeats (several million) of a family of DNA sequences 172 base pairs long. This family is referred to as α-component. A nucleotide sequence corresponding to the most abundant residue at each of the 172 positions in the repeat unit has been determined. The various repeats comprise a set of sequences which vary, in a nonrandom manner, by having alternate base pairs at one or more positions. The α-component sequences are organized into nucleosomes in chromatin. The sequence itself does not appear to instruct a unique positioning of the nucleosomal structure.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To elucidate the structure of the highly repeated set of DNA sequences in the African green monkey (Cercopithecus aethiops): this set of sequences is referred to as α-component. The set consists of sequences each 172 base pairs in length and the total number of members of the set in the genome is several million. The various members of the set are not identical, but differ from one another in one or a few of the 172 residues. We are interested in the number of members of the set, that is, the number of different sequences making up the total, the ways in which they differ from one another, the way in which they are organized within the genome in relation to one another and in relation to other genomic DNA sequences. We are also interested in studying the organization of these sequences into chromatin.

Methods Employed: DNA is isolated from tissue and from cells grown in tissue culture and purified by standard techniques. Structural analysis includes the use of restriction endonucleases and polynucleotide sequencing methods utilizing both RNA sequencing and DNA sequencing. Extensive use is made of gel electrophoresis throughout. Radioactive tracers are used. Specific enzymatic reactions are used as analytical chemical tools. DNA is also studies by means of electron microscopy. A variety of hybridization techniques, both in solution and on nitrocellulose are used. Molecular cloning of α-component sequences by recombinant DNA techniques, using an E. coli host-vector system is being initiated.

Major Finding:

Last year we reported that we had determined the nucleotide sequence of the major class of highly repetitive DNA (the so-called α-component) in the genome of the African green monkey (Cercopithecus aethiops). This 172 base pair segment is repeated several million times in the monkey genome, primarily in long tandem repeats. Because the sequence contains one site for cleavage by restriction endonuclease HindIII, the long tandem repeats can be converted to a readily isolable 172 base pair long fragment by digestion with that enzyme. The fragment can be obtained in pure form and reasonable quantity by electrophoresis on polyacrylamide or agarose gels, thus supplying material for the sequence determination. The sequence was determined, as indicated last year, by both direct DNA sequencing techniques and by procedures involving the synthesis of a cRNA copy of the DNA and then the use of RNA sequencing techniques.

Further analysis of the data described last year has revealed several new aspects of the structure of the repeating unit.

First, the sequence is not internally repetitive, but rather represents a unique 172 base pair array of nucleotides. This distinguishes this highly repetitive DNA from the characterized "satellite" DNAs of a number of other eukaryote species and indicates the existence of two classes of highly repetitive DNA, the simple (made up of multiple repeats of very short oligonucleotide segment) and the complex (such as α-component).

Second, although the DNA and RNA sequencing data allowed the unambigous and consistent assignment of a unique base pair to each of the 172 residues in the α-component monomer, it is now clear that the collection of molecules we isolated represent a set of closely related molecules and not a single molecular species. Thus, what we determined was the most abundant residue at each of the 172 positions, but not necessarily the sequence of any particular member of the set. The isolated fragments represent a mixture of molecules of identical chain length which differ by the presence of a nucleotide other than the most abundant one at one or more positions. No particular divergent member represents more than a few percent of the total. Furthermore, the divergence from the determined sequence within the members of the set is not random. That is, divergence at certain nucleotide residues occurs more frequently than divergence at other residues. The available data indicate that at least 90 percent of the molecules in the isolated set contain the most abundant nucleotide at each position, but that some positions probably contain the most abundant nucleotide in more than 99 percent of the molecules. While direct quantitation and hybridization kinetics indicate that the set as a whole occurs several million times within the monkey genome (representing from 10 to 20 percent of the total DNA of the monkey), the reiteration frequence of particular members of the set is not known.

The organization, in chromatin, of the α-component sequences has been investigated. This highly repeated DNA was shown to be packaged into nucleosomes. Furthermore, a comparison of reassociation kinetics of total DNA and nucleosomal DNA demonstrated that the α-component sequence is represented as frequently in nucleosomes as it occurs in total DNA. In addition, mononucleosomal nucleoprotein particles were isolated and, taking advantage of the known restriction enzyme sites in the α-component sequence, we were able to show that the DNA sequence does not exert a unique positioning effect on the structure of the mononucleosomal particle. Nevertheless, there does appear to be something special about the structure of the α-component sequences within chromatin. Nucleosomes containing α-component appear to be more resistant to digestion by DNase I than is total monkey DNA, but equally susceptible to digestion by Staphylococcus aureus nuclease.

Proposed Course of Research:

1. To investigate the unique structural aspects of nucleosomes containing α-component DNA. In particular, to look for proteins within monkey cells that specifically interact with α-component sequence.

2. To study further the organization of the α-component sequences within the monkey genome. Are any of these sequences interspersed with other, less frequently reiterated sequences or are they all arranged in tandem? To what DNA sequences are the α-component sequences attached both at the ends of long tandem arrays and in interspersed contexts, if such exist?

3. How are the various divergent species of the α-component monomer organized in relation to one another?

Significance to Cancer Research:

During the last few years, new methods and new approaches have permitted a start on the understanding of the organization of eukaryote genomes at the molecular level. The work we are doing is part of this large undertaking. Previous attempts to understand the nature of events related to the initiation of neoplastic disease suffered from a lack of understanding of the structure of the normal genome. With increased understanding of these complex genomes, especially those of primates, both older information and new data on the disease process itself may be elucidated.

Publication:

Rosenberg, H., Singer, M.F., and Rosenberg, M.: Highly reiterated sequences of SIMIANSIMIANSIMIANSIMIANSIMIAN. Science, 200: 394-402, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00333-15 LB

PERIOD COVERED October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

The Biochemical Basis for Defective Maturation in Myelogenous Leukemia

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHERS:	E. A. Peterson	Research Chemist Head, Protein Chemistry Section	LB NCI
	M. G. Mage	Immunochemist	LB NCI
	S. Wilson	Biologist	LB NCI
	F. Boctor	Visiting Fellow	LB NCI
	A. Torres	Research Associate	LB NCI

COOPERATING UNITS (if any)

Gilbert H. Smith, Research Biologist, C/LMBGY

LAB/BRANCH Laboratory of Biochemistry

SECTION Protein Chemistry Section

INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	2	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The main thrust of this work is to develop biochemical methods for the early diagnosis of granulocytic leukemia and methods for inducing leukemic cells to develop some or all of their functional properties as a means of partially or completely restoring host defense mechanisms in leukemia patients. Work is first aimed at identifying the main molecular constituents and metabolic processes that distinguish normal mature granulocytes from their immature precursors and establishing which of the many steps involved in granulocyte maturation are regulated by factors present in body fluids and/or by cell-mediated factors. The results will be compared with those obtained from similar studies on leukemic cells at corresponding stages of maturity in order to determine the nature and potential reversibility of the arrested maturation steps. Biochemical analyses of molecular constituents are carried out on mature and immature granulocytes isolated from blood and bone marrow by velocity sedimentation and density centrifugation procedures developed in this

(Summary cont'd)
laboratory. The effects of external cell regulators on granulocyte production and differentiation, as measured by changes in the synthesis of specific cellular components, are studied in a chemically defined culture system previously developed in this laboratory.

Methods Employed

Leukocytes are isolated from bone marrow, blood and inflammatory exudates by a variety of methods. Subcellular fractions are prepared by differential centrifugation. Proteins extracted from leukocytes and serum proteins are fractionated by column chromatography and gel electrophoresis. Glycolipids are separated by thin layer chromatography and analyzed by gas-liquid chromatography and mass spectrometry. Immunological reagents, including fluorescent antibodies, are prepared against granulocyte proteins and used to follow their formation during granulocyte maturation. Stationary tissue culture methods are employed to study the synthesis of specific proteins from radioactive precursors. The distribution of radioactive proteins in electrophoretic gels is determined by slicing the gels in a gel fractionater and counting the slices in a liquid scintillation counter. Densitometric patterns of gels stained for glycoproteins are obtained by scanning the gels in a spectrophotometer equipped with a gel scanner accessory.

Major Findings

A. Detection of Regulators of Granulocyte Production in the Bone Marrow

We are exploring the feasibility of using secondary granule glycoproteins from neutrophil leukocytes as biochemical markers in an assay for detecting regulators of granulocyte production in the bone marrow. Studies were carried out on the effects of normal and inflammatory sera on the incorporation of ^{14}C-glucosamine into the glycoproteins of bone marrow granulocytes in vitro. It was found that normal serum contains a heat-sensitive, nondialyzable factor which inhibits glycoprotein synthesis in these cells. Relative to normal serum, inflammatory serum had a marked stimulatory effect on ^{14}C-glucosamine incorporation into bone marrow cells in vitro. This property of inflammatory serum reached a maximum at about 8 hours after the initiation of inflammation in vivo and began to decrease by 24 hours. Evidence has been obtained which indicates that the stimulatory effect of inflammatory serum is probably due to the appearance, during inflammation, of a serum factor that blocks the effect of the inhibitor in normal serum rather than the result of a disappearance of this inhibitor from the serum.

B. Isolation and Partial Characterization of Glycolipids from the Secondary Granules and Plasma Membrane of Polymorphonuclear Leukocytes

Chloroform-methanol extracts of the secondary granule and plasma membrane fractions of polymorphonuclear leukocytes have been shown to contain

both non-polar and polar carbohydrate-containing materials. The ratio of the polar to the non-polar material was much higher in the plasma membrane than in the secondary granule fraction. The non-polar material contains at least two ceramide-like glycolipids and accounts for most of the broad band of periodic acid/Schiff-positive material which migrates at the dye front when granule and plasma membrane extracts are subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate. The polar material appears to be a single substance containing no fatty acids or sialic acid and consists of glucose, hexosamine, and an unidentified sugar. Expressed on a per mg of protein basis, the amount of this polar carbohydrate in the plasma membrane fraction was about seven times that of the secondary granule fraction.

C. Extraction and Purification of Granulocyte-Specific Glycoproteins

Work continues on the extraction and purification of specific glycoproteins from the cytoplasmic granules of granulocytic leukocytes. These proteins will be used as markers in specific immunochemical assays for regulators of granulocyte production in normal, inflammatory and leukemic bone marrow. At least eight glycoproteins ranging in molecular weight from 16,000 to 110,000 are associated with leukocyte granules. One of these proteins having a molecular weight of 75,000 is readily extracted by 0.2 M acetate buffer, pH 4.0, and has been identified as the iron-binding protein, lactoferrin. All of the other glycoproteins, except one having a molecular weight of 100,000, can be removed from the granules by extraction with the cationic detergent cetyltrimethylammonium bromide.

Significance to Cancer Research (Objective 2, Approach 3)

Understanding of normal and leukemic granulocyte maturation depends upon clarification of the molecular processes whereby the cell controls the appearance and disappearance of specific components. Such information has never been obtained at the biochemical level in a systematic fashion similar to that achieved in morphological studies of normal and leukemic granulocytes. Any biochemical differences in the maturation processes observed between normal and leukemic leukocytes will be exploited for purposes of developing techniques, presently unavailable, for the early diagnosis of leukemia and for improved treatment, especially with regard to the possibility of reversing the arrested maturation and decreased phagocytic activity of leukocytes from myelogenous leukemia.

Proposed Course of Research

The rate of production of granulocytic leukocytes in the bone marrow is regulated by a feedback control mechanism which responds to the rate of destruction of these cells in the peripheral tissues of the body. Feedback information could be of a positive (stimulator) or negative (inhibitor) type transmitted by humoral and/or cellular factors, but at present the nature of these factors is unknown. We are using the response of guinea

pig bone marrow granulocyte precursors to inflammatory stimuli as a model for studying the nature of the feedback factors involved in the regulation of granulocyte production. The humoral hypothesis for regulation will be examined by studying the effect of sera from normal and inflammatory guinea pigs on the synthesis of granule-specific granulocyte precursor proteins by intact cells in vitro. This approach is analogous to the use of hemoglobin as a marker for erythrocyte production in assays for erythropoietin. Unlike erythrocytes, which contain only one major specific protein, granulocytes contain many potential marker proteins in their granules and it is conceivable that humoral regulators might affect the synthesis of some granule proteins more than others. Our research plans are as follows:

(1) To further increase the specificity of our biochemical assay for granulocyte regulators by isolating and purifying the secondary granule glycoproteins so that antisera against these proteins can be prepared for use in specific radioimmune assays for these markers;
(2) To isolate, purify and test in vivo the regulatory factors already detected in normal and inflammatory guinea pig serum;
(3) Apply the information obtained with guinea pigs to studies of the regulation of granulocyte production in humans;
(4) To search for defects in the granulocyte regulatory system in guinea pig and human leukemias.

Publications

Evans, W. H. and Mage, M. G. Development of surface antigen during maturation of bone marrow neutrophil granulocytes. Exp. Hematol., 6:37-42, 1978.

Evans, W. H., Mage, M. G., Hsu, C. K., Himmelhoch, S. R., and Smith, G. H. Transplantable granulocytic leukemia in strain 13 guinea pigs. Cancer Res., 38:130-136, 1978.

Rao, R. B., and Evans, W. H. Isolation and biochemical characterization of nuclei from immature and mature guinea pig granulocytes. Arch. Biochem. Biophys., 186:89-97, 1978.

Appendix - NIH Contract No. 1-60-25423

Funding: General NCI Contract for FCRC - No individual project funding breakdown available.

Man Years Purchased: 1

Collaborating Contract Scientist: Dr. C. K. Hsu
Frederick Cancer Research Center
Frederick, Maryland

Major Findings

A transplantable granulocytic leukemia in strain 13 guinea pigs has been developed in collaboration with Dr. Hsu. Experiments are in progress to determine whether the abundant virus-like particles found in these cells are of significance in the etiology of this granulocytic leukemia. Preliminary results suggest that the disease can be transferred to new-born guinea pigs by a cell-free particulate fraction prepared from solid, leukemic tumors by the Moloney procedure.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00375-16 LB

PERIOD COVERED October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Homogeneity and Structure of Proteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E. A. Peterson	Head, Protein Chemistry Section	LB NCI
OTHER:	A. R. Torres	Research Associate	LB NCI
	M. G. Mage	Immunochemist	LB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Protein Chemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Methods for the fractionation and analysis of proteins are developed and applied to the purification of specific proteins for the study of their function and structure. Displacement chromatography is being developed for the fractionation of macromolecules and particles of biological interest, employing polyanions and polycations differing in molecular size and charge density as displacers. Current efforts are directed toward the isolation of proteins from the granules of guinea pig neutrophils for use as markers in studies on the regulation of the maturation of these cells.

PHS-6040
(Rev. 10-76)

Project Description

Objectives

To develop methods for the separation and analysis of proteins that will overcome present limitations and to apply these methods to the purification of specific proteins for the study of their function and structure.

Methods Employed

(a) Ion-exchange and copper chelate chromatography, gel filtration, and electrophoresis were the principal methods used for the fractionation and analysis of the major proteins in the granules of guinea pig granulocytes. The granules were prepared by differential centrifugation.

Major Findings

(a) This year emphasis has been given to the purification of some of the proteins of leukocyte granules for use as markers in studies on the regulation of the maturation of neutrophils in the bone marrow of guinea pigs (See Z01 CB 333-15 LB). The first of these proteins to be purified was lactoferrin, a glycoprotein specific to neutrophils within the bone marrow population but found in milk in much larger quantities. By means of chromatography on CM-cellulose and Sephadex G-75, a product was obtained from guinea pig milk that yielded a single band on electrophoresis in polyacrylamide gel in the presence of sodium dodecyl sulfate and had the known molecular properties of lactoferrin. An anti-serum prepared by injecting the pure lactoferrin into rabbits was monospecific when tested against acetate extracts of leukocyte granules and against guinea pig whey. However, a radioimmunoassay using ^{125}I-labeled lactoferrin and the anti-serum did not detect a net increase in lactoferrin in bone marrow granulocytes under conditions that produced a significant stimulation of incorporation of ^{14}C-labeled glucoseamine into granule glycoproteins (see Z01 CB 333-15 LB). A stimulation of the latter is a part of the inflammatory response, the segment of leukocyte regulation being studied. Doubts as to the suitability of lactoferrin were raised by a number of observations which suggested indirectly that lactoferrin, although associated with the granules, might not be linked in origin to the other granule proteins.

Initial efforts using CM-cellulose and related cation exchangers to fractionate granule proteins that had been extracted with sodium acetate at pH 4 were unsuccessful. Lactoferrin contaminated most of the protein fractions, apparently because of a tendency to bind to other proteins at relatively low salt concentrations. However, this difficulty was overcome by the use of copper-chelate chromatography, which permitted operation at a salt concentration high enough (0.5 M NaCl) to prevent the association of lactoferrin with other proteins. Elution with a decreasing pH gradient not only separated lactoferrin from the other proteins but purified two of the

latter to apparent homogeneity. However, one of these, lysozyme, is known not to be specific for the neutrophil granules and the other, a "cationic protein," may have come from eosinophils. Purification of other major proteins in the remaining fractions will be continued by conventional chromatography.

The residue obtained after successive extraction of the granules with acetate and citrate contains additional proteins. Extraction of this residue with 0.3 M lithium diiodosalicylate removed many of these. Treatment with 30 mM octyl-β-D-glucopyranoside was more selective, extracting only a few of the proteins at the high molecular weight end of the electrophoretic gel pattern given by total granule protein dissolved by heating in sodium dodecyl sulfate and 2-mercaptoethanol. Moreover, octyl-β-D-glucopyranoside offers operational advantages (no charge, low-absorbance, and ready removal by dialysis) that promise to facilitate the purification of some of these proteins. However, true molecular dispersion under conditions permitting fractionation by chromatography and electrophoresis has not yet been achieved.

(b) Work on displacement chromatography has been largely limited to technical improvements in the preparation of the CM-dextrans and refinements in chromatographic procedure.

Significance to Cancer Research (Objective 2, Approach 3)

(a) The isolation of a few suitable marker proteins from neutrophil granules and the preparation of anti-sera specific to them would greatly facilitate the detection and purification of regulating substances that control the maturation of these cells in the bone marrow.

(b) Displacement chromatography of proteins, nucleic acids, etc., promises to be of value at any scale of operation and therefore has significance to all research that involves the isolation of such substances. The displacers, unlike salt, do not interfere with electrophoresis, and protein fractions obtained by displacement can be directly subjected to electrophoresis or rechromatography without dialysis or concentration. Thus the examination of complex mixtures by a combination of chromatography and electrophoresis on a semi-micro scale is greatly expedited.

Proposed Course of Research

(a) Efforts to purify selected proteins from leukocyte granules will be continued, using new extracting agents in addition to those described above. Protein components will be selected for ultimate purification on the basis of the extent to which their synthesis or glycosylation is stimulated in inflammation as well as the quantity available and ease of purification.

(b) Displacement chromatography, as it develops, will be applied to the isolation of serum and cytoplasmic factors mediating bone marrow response to inflammation in the guinea pig. Its possible application in the fractionation of cell populations and leukocyte granules will be explored, using appropriate adsorbent matrices.

Publications

Peterson, A. E. Ion-exchange displacement chromatography of serum proteins, using carboxymethyldextrans as displacers. Anal. Biochem., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 CB05202-11 LB

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Isolation, Fractionation, and Charcterization of Native Nucleoproteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	O. Wesley McBride	Senior Surgeon	LB NCI
OTHER:	Raghbir S. Athwal	Visiting Associate	LB NCI
	Anne S. Olsen	NIH Postdoctoral Fellow	LB NCI

COOPERATING UNITS (if any)

Laboratory of Immunology, Bureau of Biologics, NIH

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Protein Chemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.5	3	2.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to develop methods for gene mapping in eukaryotic cells and for cloning small fractions of a mammalian genome. Gene transfer is detected by isolation of colonies of biologically transformed cells in selective medium after incubation of isolated metaphase chromosomes with mutant recipient cells deficient in HPRT or TK. The gene product is further characterized as chromosomal donor species. The method currently is most useful for determining close gene linkages. Nucleic acid hybridization is employed to determine the size of the transferred chromosome fragment (transgenome). The transgenome can be stabilized by integration into the chromosomal DNA of the recipient cell, and multiple integration sites exist.

Objectives

Biological transformation of cells with isolated and fractionated mammalian metaphase chromosomes as a means of genetic mapping and isolating restricted regions of mammalian genomes.

Methods Employed

Tissue culture procedures involving propagation of mammalian cells in monolayer and suspension cultures, plating in selective media, cloning, labeling with radioisotopes, selection of cell mutants, and metaphase arrest for chromosome isolation. Metaphase chromosome isolation and purification by isopycnic and velocity sedimentation (including unit gravity procedures), and evaluation of chromosomal DNA molecular weight by zonal density gradient sedimentation. Gene transfer to mammalian cells via uptake of purified metaphase chromosomes and isolation of biologically transformed colonies in selective media. Isolation, quantitation, and characterization of gene products by chromatography, electrophoresis, isoelectric focusing, and immunochemical procedures. Induction of micronuclei formation in mammalian cells and isolation of "microcells" by cytochalasin B induced enucleation of these cells. Isolation and biochemical and karyotypic analysis of somatic cell hybrids following interspecific cell fusion or fusion of "microcells" with intact cells. Karyological analysis of cells by fluorescence, phase, and bright-field photomicroscopy. Isolation of DNA and nucleic acid hybridization techniques.

Major Findings

It has previously been demonstrated in this laboratory that functional chromosomal donor genes can be stably transferred to recipient cells following the uptake of isolated metaphase chromosomes by mammalian cells in vitro. The transferred fragment (transgenome) comprises only a small fraction of an intact chromosome and the transgenome can exist in at least two states in the recipient cells. Initially, in an unstable state, there is a high frequency (about 10^{-1} per cell per generation) of loss of the transferred genes accompanied by a much lower frequency (about 10^{-5} per cell per generation) of stabilization of these genes. Ultimately a cell population is obtained in the second stage in which stable expression of the transgenome is exhibited by all cells and their progeny. The serial transfer of functional chromosomal donor genes by this process has provided evidence that stabilization of the transgenome involves its integration into the DNA of recipient cells.

Information is still required in two major areas before this technique can be optimally utilized for the investigation of a large number of problems of biological interest. The first area involves elucidation of the integration site, or sites, of the transgenome during stabilization. The second consists of precise characterization of the size of the transgenome,

or transgenomes, in a recipient cell and determination of the nature and location of the transgenome in cells prior to stabilization.

The first problem has been studied by analysis of the segregation of a stably transferred genetic marker, HPRT, with chromosomes of recipient cells. A clonal hamster cell line, resulting from chromosome-mediated gene transfer, was subcloned after stabilization of the transgenome. Micronuclei have been isolated from each of two resultant subclones by cytochalasin B induced enucleation, fused with HPRT-deficient mouse cells, and colonies isolated in a selective medium. Karyological analysis of these hybrid clones indicates that the transgenome was integrated into a large metacentric Chinese hamster chromosome in one case, whereas the integration site was a small acrocentric hamster chromosome in the other instance. Biochemical analyses in progress indicate that the small acrocentric chromosome also contains a hamster autosomal APRT locus. These results indicate that integration of the transgenome can occur at multiple sites and does not require crossing over at the homeologous locus and are consistent with similar results which have recently been obtained in Ruddle's laboratory at Yale.

In conjunction with these studies, methods have been developed for the isolation of micronuclei from both Chinese hamster and mouse cells in high yield. These procedures have considerable value for a variety of ongoing studies.

Determination of the size of the transgenome is in progress, utilizing various nucleic acid hybridization techniques. The necessary preliminary studies have been completed including conditions for isolating, shearing, denaturing, and hybridizing DNA; DNA molecular weight determinations; hydroxylapatite chromatography for separation of single stranded and double stranded DNA; $C_o t$ analysis of human DNA and of DNA from a hybrid cell line containing a single human X-chromosome in a mouse genomic background; nick translation of DNA; addition of poly dA to DNA using the enzyme terminal transferase and recovery of this DNA on oligo dT-cellulose. Since the amount of human donor DNA in gene transfer clones is very small, a two-step approach is being used to initially quantify it. The first step involves isolation of labeled human X-chromosomal unique-sequence DNA from a human-mouse hybrid cell line which contains a single human chromosome, the X-chromosome. DNA has been isolated from this cell line, sheared to 600 base-pair fragments, hybridized to $C_o t$ 1000, and unique sequence DNA isolated by HAP chromatography. This DNA is being isotopically labeled by nick translation and poly dA tails added. Unlabeled DNA drivers have been isolated in large quantities. The labeled human X-chromosomal unique sequences are being isolated by fractionation on hydroxylapatite after hybridization with unlabeled, sheared, whole genomic, human DNA in vast excess to an appropriate $C_o t$; the labeled human DNA probe is separated from unlabeled human DNA driver by chromatography on oligo dT-cellulose columns. Mouse related sequences are then removed from this labeled human DNA by HAP chromatography after hybridization with a vast excess of unlabeled

mouse DNA. The labeled human X-chromosome DNA thus isolated will then be used as a probe to determine how much of the human X-chromosome is present in the various gene transfer clones as described in the next section. Analogous procedures are underway to isolate a transcribed human X-chromosomal unique-sequence DNA probe using hybridization reactions driven by excess whole cellular RNA. The vast quantities of RNA required for this project have been isolated.

Proposed Course of Research

Nucleic acid hybridization experiments are in progress. A ^{3}H-labeled DNA probe containing only human X-chromosomal unique-sequence DNA is being isolated. This probe will be hybridized in vast driver excess with DNA isolated from unstable and stable transgenote clones and subclones containing human HPRT in a mouse background as well as DNA isolated from these same clones after loss of the human HPRT by segregation. This will permit determination of the size of the transgenome in unstable and stable transgenotes, the number of copies of the transgenome in these cells, and evidence for single or multiple X-chromosomal fragments in each case. Metaphase chromosomes, interphase nuclei, and non-chromosomal "debris" will be isolated and purified from unstable transgenotes and DNA will be purified from each fraction and hybridized with the DNA probe. This will provide evidence concerning the nature and location of the unstable transgenome, e.g. cytoplasmic, non-chromosomal nuclear, or chromosomal location. A labeled DNA probe will also be isolated from unstable and stable transgenotes and similar hybridization studies will further indicate whether autosomal fragments are also incorporated into these cells, which were selected for the presence of an X-chromosomal gene (e.g. multiple transgenomes from more than one chromosome). Labeled human X-chromosomal DNA sequences will be isolated and this probe will be used to identify human sequences in restriction digests of DNA isolated from stable and unstable transgenotes. Efforts will ultimately be made to isolate the human HPRT gene for cloning by these methods.

Radioisotopic labeled DNA probes containing only human X-chromosomal or transgenote, unique, transcribed sequences will be isolated by RNA-excess hybridization with whole cellular RNA isolated from the appropriate cell lines. This probe will be used for analyses similar to those described above. Moreover, it will also provide evidence concerning the extent of transcription of the transgenome.

CMGT clones will be analyzed for other detectible markers and particularly those which might provide readily available nucleic acid probes for analysis of restriction digests. For example, preliminary studies in collaboration with Dr. Kira Lueders suggest that transgenotes containing transferred mouse DNA may also contain several copies of the mouse A-particle gene. Preliminary studies in collaboration with Dr. Marcello Siniscales at the Sloan Kettering Institute suggest that transgenotes containing the human HPRT gene may also contain a human X-linked surface antigen.

Integration sites of the transgenome will be evaluated by several other independent approaches. One method provides identification of the integration site at the individual gene level involving the homeologous locus. Two-dimensional gel electrophoresis and autoradiography combined with antibody precipitation of HPRT will be employed to detect the presence, or loss, of the recipient species of HPRT (CRM positive) following stabilization of the HPRT gene.

Significance to Cancer Research (Objective 2, Approach 3)

Metaphase chromosome isolation and fractionation is a very promising approach for the fractionation of mammalian genes and can provide material for the investigation of control elements associated with specific sets of genes. The biological expression of these chromosomes introduced into recipient cells provides a method for mapping of mammalian genes. This method has also recently been used in two laboratories to transfer the malignant phenotype to recipient cells, thereby furnishing strong evidence for dominance of the malignant phenotype. Chromosome-mediated gene transfer provides a method for cloning relatively small fractions of a eukaryotic genome in an interspecies background. Combination of this method with nucleic acid hybridization procedures should permit isolation of specific eukaryotic genes for application with recombinant DNA techniques. This method should also afford insight relating to control and integration of eukaryotic genes. Moreover, if the product of the size of the transgenome and the number of different integration sites is relatively large (e.g. approaches genome size), this gene transfer method should prove extremely valuable for isolating DNA sequences associated with the malignant phenotype.

Publications

Athwal, R.S., and McBride, O.W.: Serial transfer of a human gene to rodent cells by sequential chromosome-mediated gene transfer. Proc. Natl. Acad. Sci. USA, 74:2943-2947, 1977.

McBride, O.W., and Athwal, R.S.: Chromosome-mediated gene transfer with resultant expression and integration of the transferred genes in eukaryotic cells. In Genetic Interaction and Gene Transfer. Brookhaven Symp. Biol. 29:116-126, 1977.

McBride, O. W., Burch, J. W., and Ruddle, F. H.: Cotransfer of thymidine kinase and galactokinase genes by chromosome-mediated gene transfer. Proc. Natl. Acad. Sci. USA, 75:914-918, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB05203-10 LB

PERIOD COVERED October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Immunochemical Purification and Characterization of Immunocytes and Their Components

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. G. Mage	Immunochemist	LB NCI
OTHER:	L. L. McHugh	Biologist	LB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Protein Chemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The goal of this project is the development of cell separation methods for the specific isolation of immune cells, particularly for varieties of antigen-reactive cells involved in cellular immune reactions, and for their subcellular fractionation in order to study the mechanisms involved in the development of immune reactivities and immune macromolecules. Antigen-reactive lymphocytes (ARC) are obtained from the tissues of normal mice and from mice immunized with allogeneic tumor cells and normal cells. Lymphoid cell suspensions containing ARC are tested for binding to the cell surface antigens of target cells attached to insoluble supports. The nonadherent cells are tested for depletion of cytotoxic effector activity by measurement of ^{51}Cr release from target cells. Graft-versus-host (GVH) activity is measured by the Simonsen spleen weight assay in neonatal mice. The mixed lymphocyte culture is used to test for stimulation of ARC by alloantigen in vitro, and for the generation of cytotoxic effector cells from their precursors.

PHS-6040
(Rev. 10-76)

Objectives

The goal of this project is the development of methods for the specific isolation of immune cells, particularly for different types of antigen-reactive cells involved in various cellular immune reactions to alloantigens, for their immunochemical characterization, and for their subcellular fractionation in order to study the mechanisms involved in the development of immune reactivities and immune macromolecules.

Methods Employed

Antigen-reactive cells (ARC) are obtained from normal mice or from mice immunized with allogeneic tumor cells and normal cells. Cell suspensions containing ARC are incubated on foam columns or on petri plates coated with allogeneic or syngeneic target cells, or with anti-immunoglobulin. Graft-versus-host (GVH) activity of non-adherent spleen cells is measured by the Simonsen spleen weight assay in neonatal F_1 mice. Cytotoxic activity is measured by ^{51}Cr release from target cells. Stimulation by alloantigen is measured by the mixed lymphocyte reaction (MLR) and by generation of cytotoxic effector cells in vitro.

Major Findings

1. We have prepared and used guinea pig antibody to mouse cytotoxic T lymphocytes and their precursors (anti-CTL) to study the contribution of this T cell lineage to the graft vs. host (GVH) reaction in vivo. When spleen cells are treated with anti-CTL there is a modest reduction (of about 30%) in total GVH activity. The majority of the affected cells appear to be those that do not adhere to allogeneic target cell monolayers. Mixtures of thymus and spleen cells are known to exhibit synergy in the production of GVH splenomegaly. This synergy has been found to be unaffected by treatment of either thymus or spleen cells with anti-CTL.

2. When thymus cells (unlike spleen cells) are treated with anti-CTL, some of the survivors do generate CTL activity on culture in vitro, indicating that this organ may contain a "pre-CTL precursor," i.e. a CTL-precursor that has not yet differentiated sufficiently to be susceptible to anti-CTL. We are further characterizing CTL precursor differentiation in the thymus in collaboration with Drs. Bonnie Mathieson and Ido Betel (I/LMI).

3. We have further characterized (in collaboration with Dr. James Mond [I/LI]) the spleen cells without surface immunoglobulin, preparatively separated on Petri plates precoated with anti-immunoglobulin, that are pre-B cells (i.e. they generate antibody forming cells on culture in vitro).

Significance to Cancer Research (Objective 2, Approach 1)

These studies on the specific separation of antigen-reactive cells relate to the mechanisms of cell-mediated tumor rejection. Knowledge of the

antigen-binding characteristics of immune cells may help in development of specific purifications of immune lymphocytes with antitumor reactivities and may aid in understanding the variation in tumor susceptibility of human beings and make possible control of immune mechanisms of tumor surveillance and rejection.

The GVH reaction is an iatrogenic complication of tumor therapy regimens that include bone-marrow transplantation. A specific separation of GVH-antigen reactive cells might aid in avoiding this complication.

Proposed Course of Research

We plan to (1) continue to develop the general cell separation methodology based on the binding of Ig-coated cells to anti-Ig-coated surfaces; (2) separate T cell subtypes, particularly CTLs and their precursors, and study their differentiation; (3) study the contributions of these cells *in vivo* immune reactions, such as graft rejection, GVH reaction, and tumor rejection; and (4) study the macromolecules involved in the binding of CTL to target cells.

Publications

Rothstein, T. L., Mage, M. G., Mond, J., and McHugh, L. L. Guinea pig antiserum to mouse cytotoxic T lymphocytes and their precursors. *J. Immunol.*, *120*:209-215, 1978.

SUMMARY STATEMENT
LABORATORY OF MOLECULAR BIOLOGY
DCBD, NCI
October 1, 1977 through September 30, 1978

The Research efforts in the Laboratory of Molecular Biology are divided into six major areas:

1. Identifying the viral gene products that initiate cell transformation and determining how such products affect cyclic AMP, cell surface protein (CSP) and collagen synthesis. Cyclic AMP, CSP and collagen have important roles in regulating the behavior of cultured cells.

2. Identification and characterization of the proteins that have a role in the movement, shape and adhesion of cultured cells and characterization of molecules that participate in the binding and internalization of molecules that control cell growth.

3. Regulation of gene activity with particular emphasis on the identification of molecules that activate genes and the determination of the structure and function of the regulatory regions of genes.

4. Studies on the biochemistry and genetics of the recombination of the E. coli chromosome with the λ chromosome, and studies on the mechanism of synthesis of various bacteriophage DNAs by E. coli enzymes.

5. Investigation of how the thyroid cell forms, stores and releases its hormone and the elucidation of factors that control its growth, differentiation and transformation into a malignant cell.

6. Investigations on mammary tumor virus and other oncornaviruses and elucidation of how these viruses are synthesized and how they change normal cells into cancer cells.

Part 1. Cell Transformation.

Gilbert Jay, R. Shiu and others have identified a protein that appears to be the product of the transforming gene (src) of Rous Sarcoma Virus (RSV). The protein p56 is very unusual in that it has a very short half-life (less than 30 min). p56 is pleiotropic in its action affecting membrane functions such as adenylate cyclase and glucose transport and nuclear events.

The mechanism by which transformation depresses cellular cyclic AMP levels is still under study. RSV and murine sarcoma virus MSV act differently; MSV transformed rat cells become unresponsive to prostaglandin E_1, whereas RSV transformed rat cells do not.

The role of cell surface protein (CSP) in controlling cell shape, adhesion and movement has continued to be studied by K. Yamada. RSV transformation decreases CSP and collagen synthesis by lowering the cellular

content of translatable mRNA for these molecules (Adams et al). This decrease has now been shown directly by visualizing the mRNA for procollagen and CSP on agarose gels (M. Sobel, S. Adams and J. Fagan). Further B. Howard has identified restriction fragments that are characteristic of cDNA made from collagen mRNA and shown that RNA from RSV transformed cells does not generate these fragments indicating procollagen mRNA levels are decreased in RSV transformed cells.

To facilitate studies on how procollagen expression is controlled in normal and transformed cells fragments of procollagen cDNA generated from procollagen mRNA have been inserted into a plasmid pBR-322 and cloned in E. coli X1776.

Michael Gottesman has identified a protein that is excreted in large amounts by transformed cells, particularly MSV transformed cells. The protein (MEP) has been purified to homogeneity and antibodies prepared to investigate its biological role.

Part 2. Control of Cell Shape and Movement.

M. Willingham has developed two new techniques to study factors controlling cell shape and movement. One is on extraordinarly sensitive method to observe fluorescent molecules in living cells by light microscopy. This method termed VIM (video intensification microscopy) has enabled our group to show that molecules such as α_2 macroglobulin, insulin and epidermal growth factor first bind to the surface of cells diffusely, and later accumulate into patches. Electron microscopic studies show these patches are over specialized regions of the membrane, coated pits, that are subsequently internalized as coated vesicles. F. Maxfield has shown that α_2 macroglobulin, insulin and EGF are all internalized by the same pathway. The internalized molecules move about in the cytoplasm by saltatory motion and do not immediately fuse with lysosomes.

M. Willingham has also used VIM to show that the formation of vacuoles in RSV transformed CEF is due to pinocytosis of medium occuring at the base of ruffles; the latter are very prominent in RSV transformed cells.

Michael Gottesman and F. Cabral have isolated a number of mutants of CHO cells with decreased adhesion to substratum. Two such mutants have altered internal proteins; neither of these proteins have previously been implicated in regulating cell shape.

K. Olden and K. Yamada have used CSP (cell surface protein) to study the role of carbohydrates on cell proteins. They find that unglycosylated CSP is rapidly degraded suggesting that the carbohydrate moieties prevent proteolysis.

Part 3. Regulation of gene expression.

B. de Crombrugghe, R. DiLauro and S. Adhya has demonstrated that two interspersed promoters regulate the expression of the galactose operon of E. coli. They have shown that the location of the gal operator is much

more distant from the initiation sites of transcription than in the lac, trp and λ operons suggesting a different mechanism for repression. S. Adhya has found that in cells which lack cyclic AMP (or CRP) more UDP-gal-epimerase (encoded by the promoter-proximal cistron) is synthesized than galactokinase (encoded by the promoter-distal cistron). This observation suggests a mechanism for attenuation of transcription acting distally to the end of the first cistron.

S. Adhya, A. Das, D. Court and M. Gottesman have isolated mutants defective in the termination factor rho that are conditional lethal for growth. Characterization of these mutants has revealed that rho and RNA polymerase interact for termination of transcription. It was also found the the rho mutation exhibits pleiotropic properties. One function which is altered in rho mutants is the membrane Mg-dependent ATPase. Evidence was obtained that the structural gene for one of the subunits of ATPase overlaps with the coding sequence of the rho protein.

M. Rosenberg, D. Court and H. Shimatake have examined the structure-function relationship of a segment in λ DNA which plays a central role in the control of lambda lysogeny. A rho-dependent terminator regulates the proportion of transcripts which extend throughout the distal neighboring genes leading in vivo to the synthesis of lytic functions. The same segment contains also a regulatory segment which controls transcription going in the opposite direction, leading to the establishment of lysogeny. Several mutants have been examined which affect the structure of this region and the regulation of lysogeny in vivo. In collaboration with M. Singer (Laboratory of Biochemistry, NCI) M. Rosenberg has analyzed the structure of a highly repetitive DNA segment of the monkey genome.

Michael Gottesman has begun genetic studies on Chinese hamster ovary cells and has isolated a variety of mutant cells. One interesting class is resistant to the growth inhibiting effects of cyclic AMP due to an alteration in the catalytic subunit of cyclic AMP dependent protein kinase (D. Evain and W. Anderson).

Part 4. Recombination and DNA Synthesis

S. Gottesman and K. Abremski have been investigating the mechanism of λ prophage excision. Based on the insensitivity of the reaction to coumermycin in vitro, it appears that excessive recombination, unlike integrative recombination, may not utilize negatively supercoiled DNA as a substrate.

S. Wickner has been investigating DNA replication proteins of E. coli in vitro. One of these, the dnaG protein catalyzes the synthesis of sequence specific oligonucleotides that serve as primers for DNA elongation. Six other proteins, including dnaB and dnaC proteins function prior to dnaG activity presumably by creating a dnaG recognition site. Interactions of these proteins with each other, nucleotides, and DNA have been studied.

Part 5. Thyroid Cell Function

S. Wollman has continued studies of thyroid hyperplasia. J.P. Herveg found that enlargement of capillaries and capsule were the major changes in compartment volume that occurred during the development of the hyperplasia. The mechanism of the capillary enlargement was largely by fusion of neighboring capillaries and the ultrastructure of this process was clarified by L.E. Ericson.

L. Nitsch has been propagating thyroid epithelium in vitro. He has developed a suspension culture of clusters of thyroid epithelial cells in follicular arrangement in which the cells remain differentiated and responsive to thyroid stimulating hormone.

Part 6. Mouse Mammary Tumor Virus

G. Smith and T. Henry have shown that the DNA found in introcytoplasmic A particles of mouse mammary tumor virus (MMTV) is not complementary to viral RNA and has a large degree of complexity. Thus the role of this DNA in MMTV is still obscure. The A particles do have antigenic determinants to proteins found in the MMTV virion (p27, p23,p14 and p10). G. Vlahakis has continued his studies on the genetic factors controlling mouse mammary tumor virus expression.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08000-08 LMB

PERIOD COVERED
October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)
Regulation of Gene Activity by cAMP and cGMP

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ira Pastan, Chief, LMB	LMB NCI
	Benoit deCrombrugghe, Head, Gene Regulation Section	LMB NCI
Others:	M. Sobel, Research Associate	LMB NCI
	T. Yamamoto, Visiting Fellow	LMB NCI
	J. Fagan, Guest Worker	LMB NCI
	D.Schlessinger, University of Illinois	
	V. Machia, University Naples, Italy	
	J. Krakow, Hunter College (CUNY)	

COOPERATING UNITS (if any)
University of Naples, Naples Italy
University of Illinois, Hunter College (CUNY)

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, National Institutes of Health, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.6	3.6	1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Rous Sarcoma Virus (RSV) transformed CEF contain decreased quantities of mRNA for procollagen and cell surface protein (CSP). Procollagen gene sequences from embryonic chicken bone have been introduced into plasmid PBR322 and replicated in E. coli. CSP mRNA has been partially purified.

PHS-6040
(Rev. 10-76)

Serial No. ZO1 CB 08000-08 LMB
1. Laboratory of Molecular Biology
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
October 1, 1977 through September 30, 1978

Project Title: Regulation of Gene Activity by cAMP and cGMP

Previous Serial Number: ZO1 CBD 08000-06 LMBGY

Principal Investigator: Ira Pastan

Other Investigators: B. deCrombrugghe, D. Schlessinger, J. Krakow, V. Macchia, M. Sobel, T. Yamamoto, and J. Fagan

Cooperating Units: University of Illinois
University of Naples, Naples, Italy

Man Years: October 1, 1977 through September 30, 1978

Total:	4.6
Professional:	3.6
Others:	1

Project Description:

Objectives: Cyclic AMP is required for the synthesis of a variety of enzymes in E. coli, and in animal cells. In E. coli cyclic AMP acts at the transcriptional level. Our objective is to determine at the molecular level how cyclic AMP, cyclic AMP receptor protein (CRP) and other factors control gene activity in E. coli and to use the results of these studies to determine how cyclic AMP and other factors control gene activity in animal cells.

Methods Employed: Cyclic AMP Receptor (CRP) has been purified in large amounts. Its structure is being determined by protein sequencing methods. The messenger RNA for procollagens and cell surface protein has been purified by conventional methods from cultured chick embryo cells and embryonic bone.

Major Findings: Studies on the sequence of CRP are being continued. A large insoluble fragment that has caused sequencing difficulties previously has been identified as the amino terminal fragment. The sequence of this fragment is nearing completion.

Using a wheat germ translation system RSV transformed CEF have been found to have less mRNA for procollagen and CSP. Further the amount of collagen RNA is decreased when analyzed by analysis of restriction fragments generated from partially purified collagen mRNA by reverse transcriptase and DNA polymerase. Fragments of cDNA generated from collagen mRNA

purified from embryonic bone have been inserted into plasmid PBR322 and E. coli clones containing such plasmids isolated and shown to contain collagen specific DNA sequences.

The mRNA for CSP has been partially purified from CEF.

We conclude that the RSV src gene product specifically decreases the synthesis of procollagen and CSP in animal cells by altering the accumulation of mRNA for these proteins.

Significance to Biomedical Research and the Program of the Institute: National Cancer Plan Objective 2, Approach 3. As we understand in detail how the expression of bacterial genes is controlled we will use this information to understand and then control the expression of the genes of animal cells and perhaps modify the behavior of cancer cells.

Proposed Course: To complete the sequence of CRP and determine which region interacts with DNA at promoter sites. To use the plasmids containing collagen RNA sequences to determine how the src gene product affects transcription of specific mRNAs.

Publications:

Adams, S.L., Sobel, M.E., Howard, B.H., Olden, K., Yamada, K., de Crombrugghe, B., and Pastan, I. Levels of translatable mRNAs for cell surface protein, collagen precursors and two membrane proteins are altered in Rous sarcoma virus-transformed chick embryo fibroblasts. Proc. Natl. Acad. Sci. USA 74: 3399-3404, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08001-08 LMB

PERIOD COVERED

October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

Role of Cyclic AMP and Transforming Viruses in the Regulation of Cell Behavior.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ira Pastan, Chief, LMB	LMB NCI
Other:	Peter Davies, Surgeon	LMB NCI
	Gilbert Jay, Cancer Expert	LMB NCI
	Robert Shiu, Guest Worker	LMB NCI

COOPERATING UNITS (if any)

Pediatric Oncology Branch DCT

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, National Institutes of Health, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.3	1.8	.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The product of the src gene of the Schmitt-Ruppin strain of Rous Sarcoma Virus has been shown to be a 56,000 molecular weight peptide which has an extremely short-half life (<30 minutes). Its action on cyclic AMP metabolism is under study. By protease treatment filamin, a phosphoprotein affected by cyclic AMP, has been converted to a form that no longer aggregates with itself but still binds to actin.

PHS-6040
(Rev. 10-76)

PHS-NIH
Individual Project Report
October 1, 1977 through September 30, 1978

Project Title: Role of Cyclic AMP and Transforming Viruses in the Regulation of Cell Behavior.

Previous Serial Number: ZO1 CBD 08001-07 LMBGY

Principal Investigator: Ira Pastan

Other Investigators: Peter Davies, Robert Shia, Gilbert Jay

Cooperating Units: Pediatric Oncology Branch, DCT, NCI.

Man Years: October 1, 1977 through September 30, 1978

Total:	2.3
Professional:	1.8
Other:	.5

Project Description:

Objectives: To understand the role of cyclic AMP and other protein factors in malignant transformation and particularly how these factors regulate the growth, morphology, and other properties of cultured fibroblastic cells.

Methods Employed: Cell culture, viral transformation and standard biochemical analyses of the enzymes involved in cyclic AMP metabolism and the synthesis of macromolecules.

Major Findings: Antisera which react with the product of the src gene of RSV have been prepared. Using these sera we have shown that the src gene product is a polypeptide with a MW of 56,000. The protein CP567 has a very short-half life (30 min) and is not a phosphoprotein.

Filamin: The factors regulating the polymerization of filamin have been studied: A fragment of filamin is generated by treatment with Ca activated protease that still binds to actin but no longer polymerizes. A peptide containing the major site of phosphorylation of filamin has been identified. The same site is phosphorylated *in vivo* and *in vitro* by cyclic AMP dependent protein kinase.

Significance for Cancer Research and the Program of the Institute: National Cancer Plan Objective 3, Approaches 1, 2 and 5; Objective

4, Approach 2; Objective 6, Approach 3. Various aspects of this work will lead to a better understanding of how cells become cancer cells and how the growth of cancer cells is controlled. It also has therapeutic implications.

Proposed Course: Purify p56 and determine how it acts to transform cells. Continue to investigate the effect of phosphorylation on the function of filamin.

Publications:

Davies, P., Bechtel, P., and Pastan, I.: Filamin inhibits actin activation of heavy meromyosin ATPase. FEBS Letters 77: 228-232, 1977.

Schlessinger, J., Barak, L.S., Hammes, G.G., Yamada, K.M., Pastan, I., Webb, W.W., and Elson, E.L.: Mobility and distribution of a cell surface glycoprotein and its interaction with other membrane components. Proc. Natl. Acad. Sci. USA 74: 2909-2913.

Klein, I., Moore, L., and Pastan, I. Effect of liposomes containing cholesterol on adenylate cyclase activity of cultured mammalian fibroblasts. Biochim. Biophys. Acta 506: 42-53, 1978.

Yamada, K.M., Schlesinger, D.H., Kennedy, D.W., and Pastan, I. Characterization of a major fibroblast cell surface glycoprotein. Biochemistry 16: 5552-5559, 1977.

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Davies, P., Wallach, D., Willingham, M.C., Pastan, I., Yamaguchi, M., and Robson, R.M. Filamin-actin interactions: dissociation of binding from gelation by Ca^{+2}-activated proteolysis. J. of Biol. Chem. June, 1978.

Jay, G., Shiu, R.P.C., Jay, F.T., Levine, A.S. and Pastan, I. Identification of a transformation-specific protein induced by a Rous sarcoma virus. Cell 13: 527-534, 1978.

Wallach, D., Davies, P., and Pastan, I. Purification of mammalian filamin: similarity to high molecular weight actin binding protein in macrophages, platelets, fibroblasts and other tissues. J. of Biol. Chem. June, 1978.

Wallach, D., Davies, P., Bechtel, P., Willingham, M., and Pastan, I. Volume 9 of Advances in Cyclic Nucleotide Research (Raven Press), 1978.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 CB 08006-07 LMB

PERIOD COVERED

October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

Control of Gene Expression in the E. coli K12 gal operon and Bacteriophage λ.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Sankar L. Adhya, Geneticist	LMB NCI
	Donald L. Court, Biologist	LMB NCI
	Asis K. Das, Visiting Associate	LMB NCI
	Max E. Gottesman, Head, Biochemical Genetics Section	LMB NCI
Others:	Sandra Hilliker, Staff Fellow	LMB NCI
	Meher Irani, Visiting Fellow	LMB NCI

COOPERATING UNITS (if any)

NONE

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Biochemical Genetics

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.0	6.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have continued our study of the two promoters in the gal operon. Both promoters are inducible by galactose, and operator constitutive mutants are found that allow expression of either or both in the absence of inducer. cAMP and its receptor protein are required to activate one promoter (P_I) whereas they act to prevent expression of the other (P_{II}). Expression of P_I yields equal production of all three enzymes in the operon whereas expression of P_{II} yields much greater expression of the promoter proximal gene.

In our study of transcription termination by Rho factor, we have determined that termination in vivo and in vitro occur at identical sites on λ ($t_{R}1$). Rho recognizes RNA polymerase at these sites since both polymerase and Rho mutant proteins can interact in an allele specific way to affect the termination.

We have also expanded our study of the pleiotropic defects of the rho mutations, the role of gene N of λ in transcriptional anti-termination, and the effects of λ gene expression on host cell physiology.

PHS-6040
(Rev. 10-76)

Serial No. ZO1 CB 08006-07 LMB
1. Laboratory of Molecular Biology
2. Biochemical Genetics Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
October 1, 1977 through September 30, 1978

Project Title: Control of Gene Expression in the E. coli K12 gal operon and Bacteriophage λ.

Previous Serial Number: ZO1 CBD 8006-06 LMBGY

Principal Investigators: Sankar L. Adhya
Max E. Gottesman
Donald L. Court
Asis K. Das

Other Investigators: Sandra Hilliker
Meher Irani

Cooperating Units: None

Man Years: October 1, 1977 through September 30, 1978

Total:	6.0
Professional:	6.0
Others:	0.0

Project Description:

Objectives: The gal operon of E. coli is normally subjected to both a positive control and a negative control by cyclic AMP and its receptor protein. The operon may also be subjected to a different type of control whereby expression of distal genes is regulated by the amount of transcriptional read through at various termination signals located within the operon. Our object is to analyze the control signals--initiation and termination-of the gal operon and identify the regulatory molecules that interact with these signals. We are also studying the expression of genes in temperate bacteriophage λ and their effect on the transformation of host cell physiology and gene expression.

Mutation in transcription termination factor (rho) gene affected a number of other cellular proteins. We are investigating the possibility of an overlap of genetic information in the rho locus.

Methods Employed: Standard microbial genetic and biochemical techniques.

Major Findings:

(1) The Mg-Ca dependent ATPase of E. coli and the Rho ATPase appear to share a common subunit as judged by 2-D analysis of their tryptic peptides. This explains (in part at least) the pleiotropy of the rho mutations. Other host cell ATPases may also contain this subunit.

(2) One rho mutation (rho 026) confers the property of enhanced polypeptide degradation (proteinase) activity.

(3) Mutations in RNA polymerase affect termination. These effects are dependent upon specific changes (mutations) in the Rho protein. Thus a strong argument is built for polymerase and Rho protein interactions.

(4) Termination by Rho and RNA polymerase has been shown by sequence analysis of the 3'OH end RNA to occur at the identical site (tR_1) on λ DNA in vivo and in vitro. Also the same factors (mutations, etc.) that affect termination at tR1 in vitro also affect termination the same way in vivo.

(5) N product has been shown to overcome both Rho-Independent as well as Rho-dependent termination events in vivo on λ.

(6) In vivo gal expression from P_I yields nearly equimolar amounts of gal kinase, transferase, and epimerase. Expression of P_{II} yields enhanced levels of epimerase and lowered levels of kinase. This is an example of natural operon polarity.

(7) The λ gene hin causes a reduction of cAMP retention as well as of amino acid and nucleoside (deoxyribo and ribo) uptake by the cell. Additionally the level of the regulatory nucleotide ppGpp is increased. Hin function also increases the stability of its own RNA. When expressed constitutively, Hin^+ cells, in contrast to cells that are deleted for the gene, have difficulty growing on defined medium. The gene has been precisely located by deletions but as yet no hin point mutations exist.

(8) The expresison of λ prophage replication genes (O & P) are normally incompatible with cell viability even under N^- conditions. Deletions of the N^- prophage beyond the replication genes leads to cell survival even though low level expression of O & P exists. When the prophage is N^+, high level expression of O and P kills this deletion prophage.

Significance for Cancer Research and the Program of the Institute: National Cancer Plan Object 3, Approach 1. In cancer cells, the expression of some genes are permanently turned on, i.e. expressed constitutively. Our studies are aimed to understand the molecular basis of how genes are turned on and off. We are using gal and λ as model systems. This understanding might help to prevent conversions of cells to those capable of forming cancers.

Proposed Course: 1. To determine the complete structure of the gal operon. 2. To identify all the regulatory molecules involved in turning on and off the various genes of the gal operon and phage λ. 3. To understand the biochemistry of the initiation and termination signals of gene expression. 4. To understand the transformations caused by phage growth on its host. 5. To study the structure of rho gene.

Publications:

Shapiro, J., Adhya, S., and Bukhari, A.: Introduction: New Pathways in the evolution of chromosome structure. DNA Insertion elements, Episomes and Plasmids, Cold Spring Harbor Laboratory, N.Y., 1977, pp 3-11.

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Weisberg, R.A., Gottesman, S., and Gottesman, M.E.: Bacteriophage λ: The Lysogenic Pathway. Comprehensive Virology, Vol. 8 (ed.) Heinz Fraenkel-Conrat and Robert R. Wagner, Plenum Press, 1977.

Merril, C., Gottesman, M., Court, D., and Adhya, S.: Discoordinate Expression of the E. coli gal operon after Prophage Lambda Induction. J. Mol. Biol., (1978) 118, 241-245.

Rosenberg, M., Court, D., Shimatake, H., Brady, C., and Wulff, D.: The Relation between Function and DNA Sequence in an Intercistronic Regulatory Region in Phage λ. (1978)Nature 272, 414-422.

Levine, A., Moreau, P., Devoret, R., Adhya, S., Gottesman, M., and Das, A.: Expression of Bacterial Gene Turned on by a Potent Carcinogen. Mutation Research, (1978) 50, 29-35.

Das, A., Court, D., and Adhya, S.: Pleiotrophy of Rho Mutations in E. coli. in "Molecular Basis of Virology" ed. M. Chakravorty, (1978) in Press.

Adhya, S., and Gottesman, M.: Transcription Termination. Ann. Rev. Biochemistry, vol. 47 (1978) in Press.

Kourilsky, P., Perricaudet, M., Gros, D., Garapin, A., Gottesman, M., Fritsch, A., and Tiollais, P.: Description and Properties of Some ECO RI Vectors Derived from Bacteriophage Lambda. Biochimie, (1978) in Press.

Rosenberg, M., Court, D., Shimatake, H., Brady, C., and Wulff, D. (1978) Structure and Function of an Intercistronic Regulatory Region in Bacteriophage Lambda. The Operon, Cold Spring Harbor, in Press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08010-04 LMB

PERIOD COVERED

October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

The Morphologic Mechanisms of Motility, Endocytosis, and Malignant Transformation in Cultured Cells.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Mark C. Willingham, Senior Investigator	LMB NCI
Others:	Ira H. Pastan, Chief, LMB	LMB NCI
	Fred Maxfield	LMB NCI
	Josef Schlessinger	LMB NCI

COOPERATING UNITS (if any)

Endocrinology Branch

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, National Institutes of Health, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	4	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Transformation produces many changes in cell physiology. To study these changes, we developed two new techniques: 1) a new procedure for electron microscopic localization of labelled antibodies with good morphologic preservation (EGS procedure) and 2) a new technique using intensifier target video cameras to observe the fate of small numbers of fluorescently-labeled molecules or high magnification light microscopic detail in living cells. With these new tools, we have studied receptor-mediated endocytosis, using the uptake of α_2-macroglobulin (α_2M) as an example. We have found that hormones such as insulin and EGF (epidermal growth factor) are internalized by receptor-mediated uptake in the same vesicles as α_2M. These vesicles move about in the cytoplasm by saltatory motion and do not immediately fuse with lysosomes. By electron microscopy, these vesicles are the "coated" class of endocytic vesicles and by antibody localization, α_2M reaches the lysosomal population after many hours.

PHS-6040
(Rev. 10-76)

Serial No. ZO1 CB 08010-04 LMB
1. Laboratory of Molecular Biology
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
October 1, 1977 through September 30, 1978

Project Title: The Morphologic Mechanisms of Motility, Endocytosis, and Malignant Transformation in Cultured Cells

Previous Serial Number: ZO1 CBD 8010-03 LMBGY

Principal Investigator: Mark C. Willingham

Other Investigators: Ira H. Pastan, Chief, LMB
Fred Maxfield
Josef Schlessinger

Cooperating Units: Endocrinology Branch

Man Years: October 1, 1977 through September 30, 1978

Total:	4
Professional:	4
Others:	0

Project Description:

Objectives: To investigate the mechanisms that control cell motility, receptor-mediated endocytosis, and the morphologic and growth manifestations of malignant transformation.

Methods: Cell culture, specialized light and electron microscopic morphologic and immunohistochemical methods, including the EGS ultrastructural localization procedure and video intensification microscopy (VIM).

Major Findings: Transformation of cultured fibroblasts alters their shape, adhesion to substratum, surface microstructure, agglutinability by plant lectins, and organization of cytoplasmic contractile proteins, as well as growth properties. These former properties, referred to as "the morphologic phenotype of transformation" are primarily controlled by adhesion to substratum and we have previously shown that these are unrelated in a causal manner to growth control.

To study the mechanisms that regulate adhesion and motility in cultured cells, we needed a method of morphologically localizing the proteins involved in these processes within the cell. Using antibodies to proteins purified from cultured cells, we have studied the light microscopic distribution of contractile proteins and other cellular elements. Ultrastructural localization with good morphologic preservation was considerably more difficult and no practical technique was available for this study. As a result, we undertook a long developmental study of this area and have developed a new procedure (EGS procedure) which provides specific ultrastructural

antibody localization of intracellular antigens with good morphologic preservation. Using this method, we are studying the location and relationships of the major contractile proteins in cells (myosin, actin, and filamin) to understand the mechanism of their interaction and resulting motility of the cell and its internal organelle movements.

Another technique which we have developed that provides new insights into the mechanisms of cell function is the use of a low light level intensifier video camera and taping system referred to as "Video Intensification Microscopy". This method allows the direct visualization of small amounts of fluorescently-labeled proteins in living cells, as well as the use of low intensities of light for regular phase microscopic observations at high magnification.

Alpha$_2$-macroglobulin (α_2M) is a large (780,000 MW) serum protein which acts as a broad spectrum protease inhibitor. Because of its anti-protease properties, we felt it might have some role in maintaing normal growth in cultured cells which routinely are grown in sera that contain active α_2M. Using fluorescently-labeled α_2M, we found that this molecule is selectively taken up by cultured fibroblasts. α_2M was also shown to bind to the cell surface in a specific, competable manner and its receptor showed mobility in the plane of the surface membrane in a fashion similar to the receptors for other proteins, such as insulin. These receptors were shown to be diffusely distributed on the cell surface, but rapidly clustered upon addition of α_2M. Further studies showed that these molecules accumulated in small vesicles that formed at the cell surface, and that these vesicles moved about in the cytoplasm by saltatory (specific, non-random, discontinuous) motion, similar to other organelle motion in the cell.

Using α_2M as a marker for these vesicles, double-labelled experiments were conducted with fluorescent derivations of insulin and EGF (epidermal growth factor). These experiments clearly showed that these hormones were internalized in the same vesicles as α_2M and showed the same intracellular motion properties. Further studies using electron microscopic markers showed that α_2M, and thus these other hormones, were selectively taken up in the "coated" vesicle population from the cell surface, the same vesicles which also are responsible for the uptake of low density lipoproteins (LDL) from serum. Thus, a common, single mechanism for receptor-mediated endocytic uptake emerged from these studies. These vesicles eventually transfer their contents into the lysosomal population, as seen by both fluorescent-α_2M and by ultrastructural localization of antibodies to α_2M by the EGS procedure. Since α_2M is an inhibitor of proteases, its uptake may in some way regulate the free protease activities in the lysosomal compartment, or function to protect the other proteins taken up in coated vesicles from the action of proteases. The concept that the intracellular role of hormones might be dependent on their uptake in this fashion provides new insights into the mechanisms of their action.

Other experiments using the VIM method to study the high magnification detail of phase microscopic images have shown new aspects of organelle motion and interaction. The internalization of components from the medium by non-specific endocytosis (pinocytosis) has shown that these uncoated vesicles often suffer a rapid demise by interaction with lysosomes. The tearing motions of the lysosomal-pinosomal interaction has been termed "piranhalysis". This mechanism is clearly reserved for the uncoated vesicle population and has never been observed with the coated vesicles containing receptor-mediated,

specific endocytosis molecules. These observations perhaps provide some insight into the mechanism by which cells distinguish between specific and non-specific endocytosis and determine different fates for the two pathways.

A further observation has been the origin of the vacuoles that are seen after transformation with either Bryan HT or Schmidt-Ruppin strains of Rous sarcoma virus. Using temperature-sensitive derivatives of these viruses, we have found that these cells suddenly increase their surface ruffling activity after shifting to the permissive (transformed) temperature. This surface ruffling is the origin of macropinosomal incorporation, and in these cells the ruffles clearly result in the accumulation of large numbers of pinosomes, which eventually fuse to form these prominent vacuoles.

Significance for Cancer Research and the Program of the Institute: National Cancer Plan Objective 6, Approach 3.

It appears that transformation of cultured cells in vitro is closely linked to the ability to form malignant tumors in vivo. By understanding the specific properties and their mechanisms in vitro that are essential for these altered growth properties, one might be able to control the growth and spead of tumors in vivo. The role of growth-stimulating hormones and their mechanism of action may provide significant insights into the manner in which transforming viruses use single, small polypeptide molecules to alter growth and change multiple morphologic properties.

Proposed Course: We will study the morphologic mechanisms of the action of contractile proteins, growth-regulating hormones and transforming viral proteins in cultured fibroblasts. All of these areas relate to the basic mechanisms by which central regulatory processes are altered after transformation to result in loss of growth control and the "morphologic phenotype of transformation".

Publications:

Pastan, I., Willingham, M., Anderson, W., and Gallo, M. (1977) Localization of Serum-Derived α_2Macroglobulin in cultured Cells and Decrease after Moloney Sarcoma Virus Transformation. Cell 12: 609-617.

Willingham, M.C., and Pastan, I. (1978) The Visualization of Fluorescent Proteins in Living Cells by Video Intensification Microscopy (VIM) Cell 13: 501-507.

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Willingham, M.C., Yamada, S.S., and Pastan, I. (1978). A new method for electron microscopic immunocytochemical localization of intracellular antigens in cultured cells. Exp. Cell Res. (in press).

Willingham, M.C., Yamada, S.S., and Pastan, I. (1978). Ultrastructural antibody localization of α_2-Macroglobulin in membrane linked vesicles by a new procedure in cultured cells. PNAS (in press).

Willingham, M.C., and Yamada, S.S. (1978). A Mechanism for the Destruction of Pinosomes in Cultured Fibroblasts: Piranhalysis. J. Cell Biol. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08011-04 LMB

PERIOD COVERED
October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)
Structure and Role of a Transformation-Sensitive Cell Surface Glycoprotein

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Kenneth M. Yamada, Investigator	LMB NCI
Other:	Kenneth Olden, Expert	LMB NCI
	Ira Pastan, Chief, LMB	LMB NCI

COOPERATING UNITS (if any)

Gene Regulation Section, LMB, NCI
Clinical Endocrinology Branch, NIAMD

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, National Institute of Health, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.1	2.1	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A major cell surface glycoprotein (CSP) that is involved in cellular adhesion is generally decreased after cells are established as permanent cell lines, and is depleted further after malignant transformation of fibroblasts in vitro. CSP's overall protein structure has been determined. It consists of disulfide-linked dimers and polymers of a 220,000 dalton subunit having a highly asymmetric shape. CSP's oligosaccharides are attached via asparaginyl linkages. Inhibition of this glycosylation using the new drug tunicamycin does not alter its synthesis or secretion, but results in a marked susceptibility of CSP to proteolytic degradation.

Immunofluorescence staining using affinity-purified antibody to CSP localizes it to relatively immobile fibrillar meshworks under and between cells. Treatment of cells with anti-CSP results in a morphology similar to that of many transformed cells, and a gradual antibody-induced redistribution of antigen on the cell surface.

Our objectives will be to determine the composition and structure of CSP's active site(s), and the mechanisms by which it helps to maintain normal cell behavior.

PHS-NIH
Individual Project Report
October 1, 1977 through September 30, 1978

Project Title: Structure and Role of a Transformation-Sensitive Cell Surface Glycoprotein

Previous Serial Number: ZO1 CBD 8011-03 LMBGY

Principal Investigator: Kenneth M. Yamada

Other Investigators: Kenneth Olden
Ira Pastan

Cooperating Units: Gene Regulation Section, LMB
Clinical Endocrinology Branch, NIAMD

Man Years: October 1, 1977 through September 30, 1978

Total:	3.1
Professional:	2.1
Other:	1.0

Project Description:

Objectives: A major cell surface protein (CSP) is depleted after neoplastic transformation of fibroblasts in vitro. Our objectives are to determine CSP's biochemical structure, its role in cell behavior, and its mechanism of action.

Methods Employed: CSP is isolated from chick embryo fibroblasts using urea, then further purified by salt fractionation and column chromatography at alkaline pH. CSP is quantitated by protein staining of SDS polyacrylamide gels, or by a double immunoprecipitation procedure using affinity-purified anti-CSP. The rate of processing and transport of CSP to the cell surface is determined by the rate at which newly synthesized CSP becomes trypsin-sensitive as it becomes exposed on the cell surface.

To locate inter-subunit disulfide bonds, CSP is subjected to limited proteolytic cleavage using trypsin, and the products are examined by SDS gel electrophoresis before and after reduction with dithiothreitol. Immunofluorescence is performed using either anti-CSP labeled with fluorescein, or by an indirect procedure in which anti-goat IgG labeled with fluorescein is used to locate the anti-CSP. Glycosylation of CSP is measured by incorporation of isotopically labeled glucosamine or mannose, and by the capacity of the lectin concanavalin A to bind the protein.

Major Findings: We had previously shown that isolated CSP is an adhesive protein that can restore a more normal morphologic phenotype

to transformed cells. We compared quantities of this protein on the surfaces of a variety of untransformed cells. Although it is a major protein of embryonic or adult cells placed into tissue culture, it is often decreased on permanent cell lines (although decreased further after transformation). This variability may account for some differences in morphology and alignment of untransformed cells.

The structure and amino acid and carbohydrate compositions of CSP were determined. CSP is highly asymmetric, with little evidence of secondary structure, although local domains of tertiary structure appear to exist. CSP is composed primarily of disulfide-linked dimers and polymers, with a subunit polypeptide of 220,000 daltons. The interchain disulfide bonds are confined to an end of the subunits. CSP's compostion and structure are very similar to that of the plasma protein cold insoluble globulin.

Using affinity-purified anti-CSP antibody, CSP is found to exist as fibrils under and between cells in locations consistent with an adhesive role. CSP is relatively immobile compared to other surface components as determined in collaborative experiments using fluorescence photobleach mobility methods. However, affinity-purified antibody to CSP gradually redistributes CSP to aggregates and to a "cap" similar to that found for another major fibroblast protein regulated by glucose concentrations. Treatment of living untransformed cells with anti-CSP results in a rapid change in cell shape to a morphology similar to that of many transformed cells that are deficient in CSP.

The oligosaccharides of CSP are attached via asparaginyl residues. Specifically blocking the glycosylation of CSP with the antibiotic tunicamycin does not affect its synthesis or secretion, but results in markedly increased degradation and loss of CSP. These results suggest that the role of carbohydrates on CSP is to stabilize the protein against proteolytic degradation.

Significance for Cancer Research and the Program of the Institute: National Cancer Plan Objective 3, Approach 5.

The cell surface glycoprotein CSP appears to play a major role in the maintenance of normal cellular adhesion, morphology, and cell-cell interactions, and its loss after transformation partially accounts for changes in these phenotypes in transformed cells. We have quantitated this protein in a variety of cell types, described its location and mobility, determined its overall structure and composition, and evaluated the role of its oligosaccharides in regulating its quantities on the cell surface.

Proposed Course: To determine the structure of CSP's active site(s), to evaluate the role of its carbohydrates in its biological activities, to compare its structure and biological activities with the closely related protein cold insoluble globulin, and to investigate its role in embryonic differentiation.

Publications:

Olden, K., and Yamada, K.M.: Mechanism of the Decrease in the Major Cell Surface Protein of Chick Embryo Fibroblasts after Transformation. Cell 11: 957-969 (1977).

Adams, S.L., Sobel, M.E., Howard, B.H., Olden, K., Yamada, K.M., de Crombrugghe, B., and Pastan, I. Levels of Translatable mRNAs for Cell Surface Protein, Collagen Precursors and Two Membrane Proteins are altered in Rous Sarcoma Virus-Transformed Chick Embryo Fibroblasts. Proc. Natl. Acad. Sci. USA 74: 3399-3404 (1977).

Yamada, K.M.: "Cell Morphogenetic Movements" in Handbook of Teratology, Vol. 2, pp. 199-230. J.G. Wilson and F.C. Fraser, eds., Plenum, New York (1977).

Yamada, K.M., Yamada, S.S., and Pastan, I.: Quantitation of a Transformation-Sensitive, Adhesive Cell Surface Glycoprotein: Decrease on Several Untransformed Permanent Cell Lines. J. Cell Biol. 74: 649-654 (1977).

Yamada, K.M., Schlesinger, D.H., Kennedy, D.W., and Pastan, I.: Characterization of a Major Fibroblast Cell Surface Glycoprotein. Biochemistry 16: 5552-5559 (1977).

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Schlessinger, J., Barak, L.S., Hammes, G.G., Yamada, K.M., Pastan, I., Webb, W.W., and Elson, E.L.: Mobility and Distribution of a Cell Surface Glycoprotein and Its Interaction with Other Membrane Components. Proc. Natl. Acad. Sci. 74: 2909-2913 (1977).

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Yamada, K.M., Olden, K., and Pastan, I.: Transformation-Sensitive Cell Surface Protein: Isolation, Characterization, and Role in Cell Morphology and Adhesion. Ann. N.Y. Acad. Sci. (In press) 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 CB 08012-03 LMB

PERIOD COVERED
October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

Adenylate Cyclase Activity of Transformed and Normal Fibroblasts

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Wayne B. Anderson, Research Chemist — LMB NCI

Other: Daniele Evain, Visiting Scientist — LMB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
DCBD, NCI, NIH

SECTION
Laboratory of Molecular Biology

INSTITUTE AND LOCATION
National Cancer Institute, National Institutes of Health, Bethesda, MD 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	1.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have been attempting to elucidate how the activity of the enzyme adenylate cyclase might be altered and regulated in cultured fibroblasts. Trypsin and certain other endopeptidases have been shown to markedly enhance the adenylate cyclase activity of fibroblast membrane preparations. Brief trypsin treatment of intact fibroblasts also increases cyclase activity and results in significant enhancement of intracellular cyclic AMP levels. To assess the ability of serum and certain growth promoters to influence cyclic AMP metabolism we determined the ability of these factors to alter the accumulation of cyclic AMP in response to added PGE_1. The somatomedin-like peptide MSA is found to significantly inhibit the hormone-stimulated accumulation of cyclic AMP. We have also developed a system to study cell-free hormonal desensitization of the cyclase. When isolated crude NRK plasma membranes are incubated with isoproterenol under appropriate conditions a time and hormone-concentration dependent decrease in the isoproterenol response of the cyclase system can be demonstrated.

PHS-6040
(Rev. 10-76)

Serial No. ZO1 CB 08012-04 LMB
1. Laboratory of Molecular Biology
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
October 1, 1977 through September 30, 1978

Project Title: Adenylate Cyclase Activity of Transformed and Normal Fibroblasts

Previous Serial No.: ZO1 CB 2012-03 LMB

Principal Investigator: Wayne B. Anderson

Other Investigator: Daniele Evain

Cooperating Units: None

Man Years: October 1, 1977 through September 30, 1978

Total	2.5
Professional:	1.5
Other:	1.0

Project Description:

Objectives: To investigate how adenylate cyclase activity is regulated in normal fibroblasts and how the activity of this enzyme is altered with transformation by RNA tumor viruses.

Methods Employed: Cell culture and standard biochemical analysis of adenylate cyclase activity.

Major Findings: Previously, we have shown that the properties and activity of fibroblast adenylate cyclase are altered following viral transformation and also during various stages of cell growth. To better understand the mechanisms involved in altering cyclase activity we are attempting to elucidate how this enzyme is regulated in normal fibroblasts growing in culture.

Trypsin and certain other endopeptidases can markedly enhance the adenylate cyclase activity of fibroblast membrane preparations. This activation is most pronounced when proteolysis is carried out, and cyclase activity is determined, in the presence of GTP. Brief trypsin treatment of intact NRK cells also significantly enhances adenylate cyclase activity. Again, this activation is most pronounced when cyclase activity is determined in the presence of GTP. Further, brief limited proteolytic treatment of intact NRK cells significantly enhances the intracellular accumulation of cyclic AMP, particularly in conjunction with stimulation by the hormones isoproterenol and prostaglandin E_1. The physiological relevance of proteolytic activation of adenylate cyclase remains to be determined. It is conceivable that the activity of the fibroblast enzyme is modulated by a membrane-

associated protease, or a serum protease, which in turn may be regulated by various intra- or extracellular factors.

As a sensitive means of determining a possible effect of seurm and certain growth promoters on cyclic AMP metabolism we have assessed their ability to alter the accumulation of intracellular cyclic AMP in response to added PGE_1. Serum, and a partially purified factor from calf serum, markedly decrease the PGE_1 response of intact NRK cells. The growth factors somatomedin A and MSA (a somatomedin-like peptide) also have been shown to decrease the PGE_1-stimulated accumulation of cyclic AMP in chick embryo fibroblasts. The mechanism by which these growth factors are able to modulate hormonal responsiveness requires further study.

The addition of isoproterenol to NRK cells causes a rapid, though transient, increase in the intracellular concentration of cyclic AMP. To study the mechanism for this loss of hormonal response we have developed a cell-free system for isoproterenol densensitization. When isolated NRK plasma membranes are incubated under defined conditions with isoproterenol a time and hormone-concentration dependent decrease in the isoproterenol-response of the adenylate cyclase system is noted. Occupancy of the ß-adrenergic receptor by ligand is necessary, but not sufficient, for desensitization. Cell-free densensitization exhibits an absolute requirement for ATP, GTP, and Mg^{+2}, which seems to indicate that a catalytically active adenylate cyclase is necessary to elicit the loss in hormonal responsiveness.

The adenylate cyclase system of Chinese Hamster Ovary (CHO) cells has also been characterized. A cell-free system for cholera toxin activation of CHO adenylate cyclase has been developed to elucidate conditions which regulate the stimulation of the cyclase by this toxin.

Significance for Biomedical Research and the Program of the Institute:

The intracellular concentration of cyclic AMP appears to influence many aspects of the behavior of cultured fibroblasts, as well as numerous biological processes in other cell types. Thus, it is of importance to understand how the activity of adenylate cyclase might be altered and regulated to, in turn, influence such cellular properties as growth rate and morphology. Since viral transformation also produces an altered cyclase system in some cell types, we can learn something of how this enzyme is made defective with transformation by studying the properties of the normal enzyme.

Proposed Course: We will continue to elucidate the regulatory properties of the adenylate cyclase system. Studies will be carried out to determine the mechanism by which certain proteolytic enzymes are able to activate adenylate cyclase and elevate cyclic AMP levels in intact cells. With SDS slab-gel electrophoresis we hope to be able to identify which membrane protein or proteins are modified during the activation process. Other studies will attempt to define how somatomedins and insulin alter cyclic AMP metabolism and hormonal responsiveness. Specifically, the in vitro effect of these factors on both adenylate cyclase and cyclic AMP phosphodiesterase activities under various conditions will be determined. Also, we will determine other factors or conditions which might influence the rate and extent of both desensitization and resensitization of the ß-receptor. Hormonal binding studies utilizing a labelled ß-adrenergic antagonist will be carried out to determine the presence of receptor. Other studies will deal with defining serum-free conditions for the growth of CHO cells in culture. Such

serum free conditions will be of benefit in elucidating the effect of various growth factors and hormones on these cells. The cell-free system for cholera toxin activation will be utilized to determine the effect of GTP, Ca^{++}, and calcium dependent regulator on the cyclase. Finally, myoblasts in culture will be used to determine cyclic nucleotide effects on the biological properties of these cells and to study the effect of intra- and extracellular agents and events on cyclic nucleotide metabolism with these cells.

Publications

Pastan, I., Willingham, M., Anderson, W., and Gallo, M.: Localization of serum-derived α_2 macroglobulin in cultured cells and decrease after moloney sarcoma virus transformation. Cell 12: 609-617 (1977).

Wallach, D., Anderson, W.B., and Pastan, I.: Activation of adenylate cyclase in cultured fibroblasts by trypsin. J. Biol. Chem. 253: 24-26 (1978).

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08700-06 LMB

PERIOD COVERED
October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)
Studies on the Regulation of Gene Expression in Bacterial and Animal Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Benoit de Crombrugghe	Head, Gene Regulation Section	LMB	NCI
Other:	Roberto Dilauro	Visiting Associate	LMB	NCI
	Sherrill Adams	Staff Fellow	LMB	NCI
	Mark Sobel	Research Associate	LMB	NCI
	Enrico Avvedimento	Visiting Fellow	LMB	NCI
	Taketoshi Taniguchi	Visiting Fellow	LMB	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Gene Regulation Section

INSTITUTE AND LOCATION
National Cancer Institute, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.0	1.0	4.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

(1) The control of the galactose operon in E. coli is studied as a model system for gene regulation. We have demonstrated that the regulation of this operon is exerted by two overlapping promoters P1 and P2. The cyclic AMP receptor (CRP) and cyclic AMP (cAMP) activate only P1. P2 is repressed by CRP and cAMP and is probably subject to another regulatory mechanism. We have determined the nucleotide sequence of the entire regulatory region for the wild type gal operon and the base changes in several mutants in this region. This operon constitutes an excellent model system for the study of complex genetic regulation. (2) In collaboration with Dr. Ira Pastan we have demonstrated that the levels of translatable mRNA for two large extracellular proteins, the Cell Surface Protein and Collagen, are considerably reduced in transformed cells. These two proteins play an important role in the cellular interactions between normal cells. We have also shown that the copy number of collagen mRNA sequences is severely reduced in transformed cells. We have constructed several plasmids containing portions of the collagen genes. We are studying the cause for the reduction in collagen mRNA in transformed cells and are investigating the genomic organization of the collagen genes.

Serial No. Z01 CB 03700-06 LMB
1. Laboratory of Molecular Biology
2. Gene Regulation Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
October 1, 1977 through September 30, 1978

Project Title: Studies on the Regulation of Gene Expression in Bacterial and Animal Cells.

Previous Serial No.: Z01 CB 8700-05 LMB

Principal Investigator: Benoit de Crombrugghe, Head, Gene Regulation Section

Other Investigators: Roberto DiLauro, Visiting Associate; Sherrill Adams, Staff Fellow; Mark Sobel, Research Associate; Enrico Avvedimento, Visiting Fellow; Taketoshi Taniguchi, Visiting Fellow

Cooperating Units: None

Man Years: October 1, 1977 through September 30, 1978

Total:	5.0
Professional:	1.0
Other:	4.0

Project Description:

Objective: To understand the precise molecular mechanisms which control the expression of specific genes in bacterial and animal cells.

Methods Employed: Measurements of RNA and protein synthesis in vivo and in cell-free systems; synthesis and purification of specific DNA fragments and RNA species; nucleotide sequence analysis of DNA and RNA; introduction of segments of animal and bacterial genes in the DNA of bacterial plasmids and bacteriophage; purification of factors active in regulation of gene expression.

Major Findings: 1) We use the galactose operon of E. coli as a model system to study gene regulation. When galactose is present in the medium it can serve as the principal carbon source for cellular metabolism. One of the intermediary reactions generates UDP galactose, a precursor for cell wall biosynthesis. UDP-galactose needs to be synthesized regardless of the presence of galactose in the medium or of the intracellular fluctuations of cyclic AMP. In the absence of galactose, UDP-galactose is produced from UDP-glucose in a reaction catalyzed by the enzyme UDP-galactose-epimerase, encoded by the first cistron of the galactose operon. The structure of the regulatory region of the galactose operon reflects the dual role of galactose metabolism in the cell. We have determined the nucleotide sequence of the entire regulatory region for the wild type gal operon and for a number of mutants in this region. We have

demonstrated that the regulation of this operon is exerted by two overlapping promoters (P1 and P2) subject to different regulatory mechanisms. The cyclic AMP Receptor (CRP) and cyclic AMP (cAMP) activate only P1, not P2. In the absence of CRP or cAMP trnscription initiates only at P2. CRP and cAMP inhibit transcription from p2. P2 is more active at low salt concentrations than P1. Transcripts from P2 terminate prematurely early after initiation suggesting the existence of an additional regulation. In vivo the gal repressor controls both promoters but in vitro the gal repressor only blocks initiation at P1. The gal repressor binds about 60 base pairs upstream from the initiation site for transcription. This location contrasts with the location of the operators in the lac, λ, and trp operons. In these systems the repressor binds much more closely to the initiation site for transcription and directly prevents the binding of RNA polymerase. The location of the operator in gal suggests a different mechanism for repression.

We have constructed a plasmid where part of the Tetracycline promoter has been excised and replaced by a 1000 basepair segment containing the gal promoters. The gal promoters now control tetracycline resistance. Mutants in the gal regulatory region are being generated after in vitro mutagenesis of the inserted DNA.

2) In collaboration with Dr. Ira Pastan, we are studying the changes in cellular gene expression which occur when animal cells are transformed by oncogenic viruses. Our particular interest is in the regulation of the synthesis of two important extracellular proteins which play an important role in the interaction of normal cells with their neighboring cells, Collagen and Cell Surface Protein (CSP). These proteins are present in reduced amounts in transformed cells. We sought to determine the cause for this reduction by translating total RNA from normal and Rous Sarcoma virus (RSV) transformed chick embryo fibroblasts (CEF) in a cell-free system from wheat germ. We find that transformed CEF have a 5-fold reduction in the level of translatable mRNA for Cell Surface Protein and a 10-fold reduction in translatable mRNA for collagen precursors.

Starting from an enriched preparation of collagen mRNA, purified from chick calvaria and long bones, we have synthesized double stranded complementary DNA. This synthetic DNA was digested by restriction enzymes to generate a set of unique DNA fragments. We identified these fragments as containing collagen gene sequences. The fragments are not generated with reactions using RNA from RSV transformed chick cells. Hence, the copy number of collagen mRNA sequences is severely reduced in transformed cells. These DNA fragments were ligated to the DNA of plasmid pBR322 and cloned in E coli 1776. They were also used to screen a set of hybrid plasmids containing larger inserts. The hybrid plasmids are being further characterized. Using these amplified collagen gene sequences we are investigating the cause for the decrease in the copy number in collagen mRNA sequences in transformed cells.

Significance for Cancer Research and the Program of the Institute: The E. coli galactose operon is an excellent model for complex genetic regulatory mechanism. We anticipate that similar complex controlling mechanisms regulate gene expression in animal cells. Our other studies using normal and cancerous cells compare the activity of specific genes, encoding proteins which are

important for the interactions between cells. Such studies aim at establishing the molecular mechanisms which regulate the activity of these genes.

Proposed Course: (1) Prove that the two promoters of the gal operon are activated in vivo under different physiological conditions; define the precise interaction between the controlling proteins and the DNA regulatory segment of the gal operon.; purify additional factors which regulate expression of the gal operon. (2) Using our collagen DNA probes study the synthesis of collagen mRNA in normal and in transformed cells, in differentiating tissues, in human fibroblasts isolated from patients with an altered synthesis of collagen. Clone fragments of genomic DNA containing larger portions of the collagen genes and its regulatory elements. Initiate in vitro and in vivo studies on the regulation of the collagen genes.

Publications:

de Crombrugghe, B.: DNA-Dependent Cell-Free Protein Synthesis. In Weissbach, H. (Ed): Molecular Mechanisms of Protein Biosynthesis. Academic Press, 1977.

Adams, S.L., Sobel, M.E., Howard, B.H., Olden, K., Yamada, K.M., de Crombrugghe, B. and Pastan,I.: Levels of Translatable mRNAs for Cell Surface Protein, Collagen Precursors and Two Membrane Proteins are Altered in Rous Sarcoma Virus-Transformed Chick Embryo Fibroblasts. PNAS 74: 3399-3403, 1977.

Musso, R.E., DiLauro, R., Adhya, S. and de Crombrugghe, B.: Dual Control for Transcription ofthe Galactose Operon by Cyclic AMP and Its Receptor Protein at Two Interspersed Promoters. Cell 12: 847-854, 1977.

Howard, B.H., Adams, S.L., Sobel, M.E., Pastan, I. and de Crombrugghe, B.: Decreased Levels of Collagen mRNA in Rous Sarcoma Virus Transformed Chick Embryo Fibroblasts. J. Biol. Chem. 1978 (in press).

de Crombrugghe, B. and Pastan, I.: Cyclic AMP, The Cyclic AMP Receptor Protein and Their Dual Control of the Galactose Operon. The Operon Book , Cold Spring Harbor, N.Y. Reznikoff and Miller (Eds.), 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08701-04 LMB

PERIOD COVERED

October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

An Examination of a Variety of Nucleic Acid Structural and Functional Relationships Involved in the Regulation of Cellular and Viral Gene Expression.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Rosenberg	Senior Investigator	LMB NCI
Other:	H. Shimatake	Visiting Fellow	LMB NCI
	T. McCutchen	Staff Fellow	Biochem. NCI
	K. McKenney	FAES Graduate Program	Johns Hopkins University
	C. Brady	Microbiologist	LMB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Gene Regulation Section

INSTITUTE AND LOCATION

National Cancer Institute, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have been investigating nucleic acid structure involved in the control of transcription initiation, termination, and post-transcriptional processing and modification of various RNA transcription products. Using a number of lamdoid transducing phages we have been able to examine the relationship between regulatory function and DNA sequence in several bacterial and viral operon control regions. The characterization of mutants which affect both transcriptional and translational expression in these regions has afforded information on the precise molecular events which lead to control element function.

In addition, we have used a variety of defined prokaryotic mRNAs to examine the requirements for translation of these transcripts in a eukaryotic cell-free system. Our results indicate that analogous to many eukaryotic mRNAs, efficient translation of the prokaryotic mRNAs is obtained only upon modification of their 5' termini with a 7-CH_3-guanosine "cap" structure.

In collaboration, we have also been characterizing a 172 bp DNA segment which is repeated millions of times in the genome of the African Green Monkey. These sequences are also found recombined into the genomes of certain defective variants of Simian Virus 40. Several defective virions are presently under study.

Serial No. Z01 CB 08701-04 LMB
1. Laboratory of Molecular Biology
2. Gene Regulation Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1976 through September 30, 1977

Project Title: An examination of a variety of nucleic acid structural and functional relationships involved in the regulation of cellular and viral gene expression.

Previous Serial Number: Z01 CBD 8013-03 LMBGY

Principal Investigator: Martin Rosenberg

Other Investigators: C. Brady
H. Shimatake
T. McCutchan
K. McKenney

Cooperating Units: None

Man Years: October 1, 1977 through September 30, 1978

Total:	3.0
Professional:	2.0
Other:	1.0

Project Description:

Objectives: 1) To examine the relationship between function and DNA structure involved in the control of transcription initiation, termination, and post-transcriptional modification of RNA.

2) To examine nucleic acid structure and function of IS2 type insertion elements found in the bacterial genome.

3) To examine certain highly reiterated DNA sequences in the monkey genome and their interaction with simian virus 40.

4) To investigate the ability of defined bacterial and phage mRNAs to be efficiently translated in eukaryotic cell-free systems.

5) To develop new techniques which can be applied to both these studies and to the general examination of nucleic acid structure in other biological systems.

Methods Employed: Purification of specific RNA transcripts synthesized either in vivo or in vitro; nucleic acid sequence analysis of both RNA and DNA; primed extension of RNA transcription products with deoxyribonucleotides;

isolation of protein factors involved either in the processes of transcriptional termination or post-transcriptional modification of RNA; isolation of purified DNA restriction enzymes and their employment in obtaining specific purified fragments of both viral and cellular DNAs.

Major Findings: 1) The nucleic acid primary structure at the termini of the bacteriophage T7 "early" mRNAs synthesized both in vivo and in vitro have been examined. The sequence information obtained supports a model for transcription of the "early" region in which a high molecular weight precursor RNA molecule is post-transcriptionally processed to yield monocistronic messengers. The processing agent is a host specified endonuclease, Ribonuclease III, which is also involved in the post-transcriptional processing of a ribosomal RNA precursor in E. coli. Thus, it was shown that the T7 "early" RNAs are direct products of RNase III cleavage of a large polycistronic precursor molecule.

In addition, we demonstrated that the 3'-termini of the processed RNAs contained sequence heterogeneity arising from the addition of several (up to 5) adenylate residues to the 3'-end. These A additions appear to be a non-template specified, post-transcriptional, post-processing, modification of the RNA.

In an effort to further elucidate the mode of action of RNase III in the processing event, as well as examine the entire structure of an intercistronic region of the phage, we have now isolated and sequenced an RNA fragment which traverses the region between two adjacent "early" T7 genes (thereby spanning an RNase III cleavage site).

Sequence analysis of this fragment indicates that the processing enzyme produces a single endonucleolytic cleavage in this region giving rise directly to the G0.3 and G0.7 mRNAs. In addition, the 3'-terminal adenylic acid residues observed previously on the in vivo T7 early RNA species were not found in these fragments and, therefore, must represent a post-transcriptional, post-processing modification of the RNA.

The secondary structure of this region is also quite characteristic. Two relatively stable base-paired stretches of RNA adjoin the cleavage site in a somewhat symetrical arrangement. These double-stranded regions appear to be required for recognition of the cleavage site by RNase III. The site of scission however is located in a small region of RNA set between the double-stranded regions.

2) The nucleic acid structure of the 3'-terminal regions of a number of RNA transcripts synthesized in vitro from λ and λ gal transducing phages are being examined. These RNAs represent both independently terminated transcription products as well as transcripts requiring the protein factor, Rho, for their termination. Sequence analysis at the 3'-ends of some of these products has indicated:

a) that all of the transcripts examined possess similar degrees of 3'-terminal sequence hetereogeneity which consisted predominately of the addition of 1 to 5 adenylate residues to the 3'-terminus of the transcript and b) that rho factor enhanced termination results in a definite structural change in

the nucleotide sequence with which an RNA molecule can terminate.

In addition, we have developed a new methodology for examining the nucleic acid structure in the untranscribed portion of the DNA immediately adjacent to the 3'-terminus of an RNA transcript. This technique has been applied to the determination of sequence information throughout the entire termination region of these transcription products. Little structional homology is apparent in the template DNA beyond the 3'-ends of these transcripts. The lack of homology suggests that this region might not be important to the termination process. Thus, the majority of the encoded termination signal is transcribed into RNA.

More recently we have determined the DNA sequence surrounding and examined termination at a totally rho dependent site (t_{R1}) in bacteriophage λ. The DNA composition at t_{R1} is 75% AT base pairs and termination occurs heterogeneously over four adjacent base pairs. No GC rich or U rich sequence is found at the 3'OH end of the RNA. This sequence is quite different from other transcripts that terminate independently of Rho. However, there is a stable base-paired stem and loop structure near the end of this RNA.

In the absence of Rho factor with normal transcription conditions (i.e. 37° and high triphosphate levels), RNA polymerase undergoes a substantial pause at this site. A mutation, cnc, that interferes with base pairing in the stem and loop, has two effects on transcription: RNA polymerase does not pause in the absence of Rho and transcription does not terminate in the presence of Rho. This suggests that the stem and loop induces RNA polymerase to pause, and that a paused polymerase is important for Rho action.

We have now completed a far more extensive characterization of the entire intecistronic region in which this termination site is positioned. A number of related transcriptional and translational regulatory elements have been defined in this region. More mutations affecting terminator function have been characterized. In addition, we have defined a site (NutR) located immediately preceeding the terminator which is apparently involved in anti-termination function. We have shown that the viral function, N, acts in some way to prevent termination (i.e. anti-terminate) at the t_{R1} site thereby allowing for the quantitative and temporal control of transcription through this intercistronic reigon (i.e. transcription attenuation). Our data indicate that the Nut R site compriser an ∿ 17 nucleotide sequence which displays a hyphenated two fold rotational symmetry. This site is in some way involved in N protein recognition prior to the action of N at the various downstream termination sites.

3) The c17 mutation of bacteriophage λ is known to create a new promoter site within the λ genome for transcription by the host RNA polymerase. We have determined the nucleic acid sequence and examined in vitro RNA transcription in the region within and surrounding the c17 promoter. Structural analysis indicated that the new promoter is created by a tandem DNA duplication of nine consecutive base pairs which comprise part of a rho-dependent termination signal (tR_1) in the λ genome. Polymerase protection studies indicate that the tR_1 terminator region, positioned 15-50 residues 5' to the c17 initiation site, overlaps with the c17 promoter

and possibly serves as a recognition signal for those polymerase molecules which start transcription at the c17 promoter. The 9 bp DNA duplication uniquely creates a "heptamer" sequence, TATAATT, (similar to those found in other promoter sites) at the junction of the repeat. Our studies indicate that RNA polymerase will bind efficiently at this site and initiate transcription 6 bp to the right of this heptamer sequence with a cytidine triphosphate (CTP). Although potential purine startpoints occur 4 and 8 bp from the heptamer, little if any initiation occurs from these sites. This suggests that at the c17 promoter the steric constraints on promoter function outweigh the necessity for RNA polymerase to initiate transcription with a purine triphosphate. A number of secondary mutations which effect the c17 promoter (kindly supplied by the laboratories of D. Wulff and D. Friedman) are presently under study.

4) The galactose operon of E. coli is subject to both negative control by gal repressor and positive control by cAMP and its receptor protein (CRP). We have sought to define the nucleotide sequence of the promoter-operator sites responsible for gal regulation. Analysis of transcripts of restriction fragments combined with direct DNA sequencing enabled us to derive the sequence for the gal regulatory region. Cleavage by restriction endonuclease Hin f abolishes cAMP-CRP dependent but not CRP independent transcription. Formation of a cAMP-CRP dependent preinitiation complex prevents cleavage by Hin f. Thus the region upstream of the Hin f site is important for cAMP-CRP stimulation of transcription. This region contains sequence similarities with the CRP recognition site of lac. One gal operator constitutive mutant is resistant to Hin f cleavage suggesting the operator may include at least part of the Hin f site. Further analyses are required to confirm this possibility.

5) Between 10 and 20 percent of the genome of the African Green monkey is a single highly repetitive class of DNA termed α-component. The bulk of the α-component consists of long tandem repeats of a segment that is 172 bp in length. A single endo R HindIII site occurs within most of the 172 bp repeats permitting isolation of the monomeric unit. An unambiguous sequence defining the most abundant nucleotide at each of the 172 posiitons in the monomer has been determined by both direct DNA and cRNA-fingerprint methods. However the data indicate that the many repeats of the 172 bp monomer unit are not identical. Thus, the highly repeated DNA component is a set of closely related sequences differing from one another at one or a few residues: the sequence divergence appears to be nonrandom. Neither the number of members in the set nor their relative frequency is known. The data do show that the most abundant residue at each position occurs in at least 90 percent of the members of the set. Nevertheless, it is important to note that the determined sequence may not represent the structure of any member of the set.

Some of the defective variants of SV40 that arise upon high multiplicity serial passage of virus in African Green monkey cells contain covalently linked monkey and wild type viral DNA sequences. In a large proportion

of these recombinant (substituted) variants a portion of the monkey DNA is homologous to α-component. We have compared the nucleotide sequence of the monomer of α-component with that of the homologous sequences in two independently derived defective variants. In each of the two variants the homologous sequences differ from one another in length and are less than a complete copy of the 172 bp fragment but represent overlapping portions of the monomer. Within the region of the monomer common to both defectives there are variations in sequence in a few residues, suggesting that different members of the set of sequences comprising the monomer may have been incorporated in each case. In several instances the sequence data define joints between α-component monomer sequence and other sequences. In some cases the joints between α-component monomer sequence and other sequences. In some cases the joints are with wild type SV40 DNA and in others the jointed component may represent low reiteration frequency monkey DNA.

The mechanism(s) by which the SV40-monkey recombinants arise is not understood. Both hybridization experiments and sequence data show that the monomer has no extensive homology to wild type SV40 sequences: there are however short regions of homology near the joints as well as in other regions of the monomer. These homologies as well as certain other features of the sequences surrounding the joints may be involved in the recombinational events leading to formation of defective viruses.

6) Utilizing defined bacterial and phage mRNAs transcribed in vitro from a variety of lambdoid phages, we have investigated the relationship between the modification of 5' triphosphate end of these transcripts with a 7-methyl-guanosine moiety (i.e. "cap" structure) and the translational efficiency of these prokaryotic mRNAs in wheat germ cell-free extracts. Our results demonstrate an almost absolute requirement for the "cap" structure to obtain efficient translation of the prokaryotic transcripts. The prokaryotic mRNAs containing the "cap" are, in fact, translated as or more efficiently than hemoglobulin mRNA on a per mole basis. It appears that all the structural information necessary for proper and efficient recognition and translation of prokaryotic mRNAs using eukaryotic components is encoded in the prokaryotic transcript except for the presence of the 5' 7-methyl guanosine modification. This implies strong evolutionary constraints on the RNA structure which is used for ribosome recognition and translation initiation, as well as similar constraints on those parts of the ribosome which must interact with this RNA structure.

Significance for Cancer Research and the Program of the Institute: National Cancer Plan Objective 3, Approach 1. The regulation of gene expression in both prokaryotic and eukaryotic systems involves numerous and complex structural and functional interactions between nucleic acids and proteins. It is highly probable (and experimental evidence increasingly supports the contention) that many of the nucleic acid fine-structure interactions which occur on the prokaryotic level have basic similarities to analogous type interactions known to occur in higher cells. Our studies involve the determination of the nucleic acid structure involved in the

control of the initiation and termination of transcription by RNA polymerase and the post-transcriptional processing of certain RNA species by specific endonucleases (e.g. RNase III) in both bacterial and related viral systems. The elucidation of these transcriptional processes and their regulatory elements will be a basis for understanding similar processes occurring in eukaryotic cells (both normal and transformed) and virus (oncogenic and other).

Proposed Course: To examine the mechanisms by which RNA transcripts are initiated, terminated and subsequently processed during the cellular and viral life cycle and to investigate further the structure-function relationships involved in a variety of nucleic acid-protein interactions.

Publications:

Howard, B.H., de Crombrugghe, B., and Rosenberg, M.: Transcription In Vitro of Bacteriophage Lambda 4S RNA: Studies on Termination and Rho Protein. Nuc. Acids Res. 4, 827-842, 1977.

Musso, R.E., and Rosenberg, M.: Nucleotide Sequences at Two Sites for IS2 DNA Insertion. In Bukhari, W.I. (Ed.): DNA Insertion Elements, Plasmids, and Episomes. Cold Spring Harbor Laboratory, 597-600, 1977.

Rosenberg, M., Segal, S., Kuff, E.L., and Singer, M.F.: The Nucleotide Sequence of Repetitive Monkey DNA Found in Defective Simian Virus 40. Cell, 11, 845-857, 1977.

Roberts, T.M., Shimatake, H., Brady, C. and Rosenberg, M.: The Sequence of the cro Gene of Bacteriophage Lambda. Nature 270: 274-275, 1977.

Rosenberg, M., Court, D., Wulff, D.L., Shimatake, H., and Brady, C.: The Relationship Between Function and DNA Sequence in an Intercistronic Regulatory Region in Phage λ. Nature 272, 414-422, 1978.

Kupper, H., Sekiya, T., Rosenberg, M., Egan, J., and Landy, A.: Identification and Characterization of a Rho-dependent Termination Site in the Gene Coding for Tyrosine tRNA su_3. Nature 272, 423-428, 1978.

Rosenberg, H., Singer, M., and Rosenberg, M.: Highly Reiterated Sequences of SIMIANSIMIANSIMIAN. Science 1978, in press.

Rosenberg, M., Court, D., Wulff, D., Shimatake, H., and Brady, C.: Structure and Function of an Intercistronic Regulatory Region in Bacteriophage Lambda. Cold Spring Harbor, J. Miller (Ed.) in press.

Keene, J.D., Schubert, M., Rosenberg, M., and Lazzarini, R.H.: A Comparative Study of Nucleotide Sequences at the 3' Termini of Ribonucleic Acids From Vesicular Stomatitis Virus and its Defective Interfering Particles. ICN-UCLA 1978 Meeting Persistent Viruses, Todaro, G., Stevens, J. and Fox, C.F. (Eds.) 1978, in press.

Singer, M.D., Rosenberg, M., Rosenberg, H., McCutchan, T., Wakamiya, T., and Segal, S.: Monkey DNA Sequences in Defective Simian Virus 40 Variants. ICN-UCLA 1978 Meeting, Persistent Viruses, Todaro, G., Stevens, J. and Fox, C.F. (Eds.) in press.

Shimatake, H. and Rosenberg, M.: The C17 Mutation of Bacteriophage Lambda - An Unusual Promoter. Federation Proc., 1978, in press.

Keene, J.D., Schubert, M., Lazzarini, R.A., and Rosenberg, M.: Nucleotide Sequence Homology at the 3' Termini of RNAs from Vesicular Stomatitis Virus and Its Defective Interfering Particles. PNAS 1978, in press.

Wakamya, T., McCutchan, T., Singer, M. and Rosenberg, M.: Structural Studies on Defective SV40 Variants Substituted With Monkey DNA Sequences. Federation Proc. 1978, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08702-17 LMB

PERIOD COVERED
October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

Endocytosis in the Thyroid Gland

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: S.H. Wollman	Head, Cell Organization Section	LMB	NCI
Other: J.D. Zeligs	Research Associate	LMB	NCI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Cell Organization Section

INSTITUTE AND LOCATION
National Cancer Institute, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The typical thyroid epithelial cell can take in colloid from the follicular lumen by macropinocytosis. It can also phagocytose red blood cells. We propose to study the mechanism of these processes by electron microscopy, histochemistry and related techniques.

PHS-6040
(Rev. 10-76)

Serial No. Z01 CB 08702-17 LMB
1. Laboratory of Molecular Biology
2. Cell Organization Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
October 1, 1977 through September 30, 1978

Project Title: Endocytosis in the Thyroid Gland

Previous Serial Number: Z01 CB 8702-16 LMB

Principal Investigator: Seymour H. Wollman

Other Investigators: Joseph D. Zeligs

Man Years:

Total:	0
Professional:	0
Other:	0

Project Description:

Objectives: To determine mechanism of thyroid hormone release, and more generally, endocytotic properties of typical thyroid epithelium.

Major Findings: None

Significance for Cancer Research and the Program of the Institute: This project fits most clearly under Objective 3, Approach 5, having to do with properties of the cell surface.

Proposed Course: To be resumed in the future.

Publications:

Zeligs, J.D. and Wollman, S.H.: Pseudopod behavior in hyperplastic thyroid follicles in vivo. J. Ultrastruc. Res. 60: 99-105, 1977.

Zeligs, J.D.: Ultrastructure of the degradation of erythrocytes by thyroid epithelial cells in vivo. Am. J. Pathol. 89: 85-104, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 03703-26 LMB

PERIOD COVERED
October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)
Thyroid Hormone Synthesis and Storage

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: S.H. Wollman Head, Cell Organization Section LMB NCI

Other: None

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Cell Organization Section

INSTITUTE AND LOCATION
National Cancer Institute, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has as its objectives to learn about: (1) The mechanism by which the thyroid gland maintains a concentration of iodide elevated above that of the blood, and (2) The mechanism by which the thyroid gland forms and accumulates protein-bound iodine.

The work is largely based upon in vivo studies and makes use of autoradiography, cytochemistry at ultrastructural level, kinetic studies, and inhibitors of steps in the synthesis of thyroid hormones.

PHS-6040
(Rev. 10-76)

Serial No. Z01 CB 08703-26
1. Laboratory of Molecular Biology
2. Cell Organization Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
October 1, 1977 to September 30, 1978

Project Title: Thyroid Hormone Synthesis and Storage

Previous Serial Number: Z01 CB 8703-25 LMB

Principal Investigator: Seymour H. Wollman

Man Years: October 1, 1977 to September 30, 1978

Total:	0.0
Professional:	0.0
Other:	0.0

Project Description:

Objectives: To learn about the mechanism of synthesis and storage of thyroid hormones.

Major Findings: None

Proposed Course: To be resumed in the future.

Significance for Cancer Research and the Program of the Institute: Some of the work reported here may best be put into Objective 3, Approach 5 because they give information on properties of cell surface and cell membranes.

Publications:

Haeberli, A., Millar, F.K. and Wollman, S.H.: Accumulation and localization of radiocalcium in the rat thyroid gland. Endocrinology 102: 1511-1519, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08704-26 LMB

PERIOD COVERED

October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

Production and Properties of Transplantable Thyroid Tumors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S.H. Wollman	Head, Cell Organization Section	LMB	NCI
Other:	J.D. Zeligs	Research Associate	LMB	NCI
	L. Nitsch	Visiting Fellow	LMB	NCI
	L.E. Ericson	Visiting Scientist	LMB	NCI

COOPERATING UNITS (if any)

J.P. Herveg, Faculte de Medecine, Universite Catholique de Louvain, B1200 Brussels, Belgium

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Cell Organization Section

INSTITUTE AND LOCATION

National Cancer Institute, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.0	4.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To study the thyroid growth and the production and properties of transplantable thyroid tumors in the rat: changes occurring during growth, hyperplasia and involution, growth and properties of thyroid cells and thyroid tumor cells in culture.

We are also studying a variety of types of cells just recently recognized to be present in the normal thyroid gland and which may be the cells of origin of some of the different histologic types of thyroid tumors which have been observed in the past.

PHS-6040
(Rev. 10-76)

Serial No. Z01 CB 08704-26 LMB
1. Laboratory of Molecular Biology
2. Cell Organization Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
October 1, 1977 through September 30, 1978

Project Title: Production and Properties of Transplantable Thyroid Tumors

Previous Serial No.: Z01 CB 8704-25 LMB

Principal Investigator: Seymour H. Wollman

Other Investigators: J.D. Zeligs, J.P. Herveg, L.E. Ericson and L. Nitsch

Cooperating Units: Department of Biology, Temple University
Philadelphia, Pennsylvania, and
Laboratory of Cell Biology, DCBD, NCI

Man Years: October 1, 1977 through September 30, 1978

Total:	5.0
Professional:	4.5
Other:	0.5

Project Description:

Objectives: To study thyroid growth and the production and properties of transplantable thyroid tumors in the rat: changes occurring in the thyroid during growth, hyperplasia and involution. We are also studying a variety of cells recently recognized to be present in the normal thyroid gland and which may be the cells of origin of some of the different histologic types of thyroid tumors observed in the past. We are also trying to grow normal and neoplastis thyroid epithelium in vitro.

Objectives at present: To study thyroid growth: especially changes occurring during the development of thyroid hyperplasia. We are also studying the properties of primary thyroid cultures especially of the thyroid epithelial cells.

Methods employed: Hyperplasia: Hyperplasia was produced in rat thyroid gland by feeding the goitrogen, thiouracil. Various details of the nature of the growth process were examined by light and electron microscopy.

Tissue culture: Thyroid gland was dissociated by collagenase treatment. Small clusters of epithelial cells were isolated from the dissociated tissue by differential centrifugation. Clusters were cultured while they adhered to the surface of a disk or in suspension and their properties were examined by electron microscopy.

Results: Hyperplasia (with Herveg): In the hyperplastic thyroid gland there is a gross enlargement of the capillaries associated with follicles. However, there are great regional differences. Capillaries in the capsule or in connective tissue partitions within the gland are not enlarged despite their close proximity to the follicles. There is also a gross enlargement in the thyroid capsule. It also exhibits specificity in the response since that part between the thyroid and parathyroid does not enlarge.

(With Ericson): Ultrastructural observations show that endothelial cells and pericytes in the thyroid gland have little rough endoplasmic reticulum (ER). This has been considered reasonable since the primary function of the capillary wall is to act as a barrier. However, in the hyperplastic gland there is an extensive development of the rough ER. This points up the possibility that endothelial cells and pericytes may be manufacturing a lot of membrane or substances for export. The response is specific in that the endothelium of lymphatics has little rough ER and it does not change appreciably in the hyperplastic gland.

Some aspects of the enlargement of thyroid capillaries during the development of hyperplasia has been examined especially ultrastructure of the process by which neighboring capillaries fuse and eliminate the capillary walls between the vessels. It appears that in the hyperplastic thyroid many endothelial cells project pseudopods from their abluminal surfaces. The pseudopods are short and wide and contain no vesicular organelles. About half of those observed penetrated the basal lamina even when there was no other endothelial cell nearby. In the presence of a neighboring capillary pseudopods with the same cytoplasmic matrix were found to be long and narrow. They penetrated through basal lamina and fibrous collagen and through the wall of the neighboring capillary. Occasionally there was separation of endothelial cells and stages in the formation of a fistula between neighboring capillaries could be recognized. Separation of endothelial cells sometimes exposed intercapillary collagen. This might explain aggregates of platelets frequently observed in capillary lumens.

The ultrastructure of the rapidly growing thyroid capsule was observed during the development of thyroid hyperplasia. The major finding was the presence of lymphocytes and monocytes in the capsule during the period of most rapid growth.

Mitosis (with Zeligs): The ultrastructure of mitotic cells was examined in the thyroid of young rats. This study was made as a control to our extensive observations on mitotic cells in the hyperplastic thyroid. Mitotic cells in the normal growing gland looked similar to those in the hyperplastic gland except for the apical surface. The apical surface of the mitotic follicle cell in the hyperplastic gland is fairly undistinguished whereas that in the normal gland almost invariably has a large cytoplasmic projection extending into the lumen. The structure contains cytoplasmic vesicles and is clearly not like an interphase bleb, but it may resemble what happens during capping.

Tissue culture (with Nitsch): Small clusters of typical thyroid epithelial cells can be isolated from thyroids. They are essentially free of fibroblasts. They attach to tissue culture dishes and spread but in doing so they rapidly lose their rough ER. The clusters can be maintained

in suspension culture and in this circumstance form follicles with colloid in the lumen, and in this condition retain their rough ER. The suspended follicles are responsive to thyroid stimulating hormone and to dibutyryl cyclic AMP.

Proposed Course: I plan to continue the above studies of hyperplasia.

Publications:

Wollman, S.H. and Hilfer, S.R.: Embryologic origin of various epithelial cell types in the thyroid gland of the rat. Anat. Red. 189: 467-478, 1977.

Wollman, S.H. and Hilfer, S.R.: Embryologic origin of the various epithelial cell types in the second kind of thyroid follicle in the C_3H mouse. Anat. Rec. 191: 111-122, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08705-02 LMB

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Genetic and Biochemical Analysis of Cell Behavior

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M.M. Gottesman	Senior Investigator	LMB	NCI
Other:	F. Cabral	Senior Staff Fellow	LMB	NCI
	P. Milhaud	Visiting Fellow	LMB	NCI
	G. Vlahakis	Research Biologist	LMB	NCI
	D. Evain	Visiting Fellow	LMB	NCI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Gene Regulation Section

INSTITUTE AND LOCATION
National Cancer Institute, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	3.5	)

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are utilizing the Chince Hamster Ovary (CHO) fibroblast grown in vitro to study the genetics and biochemistry of some aspects of the behavior of cultured cells. Our work has emphasized cell adhesion, morphology, response to cyclic AMP, and the role of cell surface antigens and receptors in cell behavior. We have isolated a variety of different mutants with abnormal adhesion, abnormal morphology or abnormal response to cyclic AMP and some of these have been characterized as to their biochemical defects. We are currently using specific antisera and complement to isolate CHO mutants which have lost specific cell surface antigens or are unable to secrete specific antigens and are establishing general procedures for the isolation of mutants lacking specific cell surface receptors.

PHS-6040
(Rev. 10-76)

Serial No. ZO1 CB 08705-02 LMB
1. Laboratory of Molecular Biology
2. Gene Regulation Section
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
October 1, 1977 to September 30, 1978

Project Title: Genetic and Biochemical Analysis of Cell Behavior

Previous Serial No.: ZO1 CB 08705-01 LMB

Principal Investigators: Michael M. Gottesman

Other Investigators: Fernando Cabral, Pierre Milhaud (beginning October, 1977), George Vlahakis, and Danielle Evain

Cooperating Units: None

Man Years: October 1, 1977 through September 30, 1978

Total:	3.5
Professional:	3.5
Other:	0.0

Objectives: To determine by genetic manipulation the cell proteins needed to maintain cell adhesion, morphology, response to cAMP, and normal receptor function.

Methods: Cell culture; isolation of cell behavior mutants; somatic cell hybridization; immunologic techniques including immunoprecipitation and complement-mediated lysis of nucleated cells; electrophoretic and chromatographic analyses.

Major Findings:

(1) We have isolated two classes of mutants which are resistant to the morphologic and growth inhibitory effects of increased levels of cyclic AMP. One class has a mutant cAMP dependent protein kinase, indicating that these effects of cAMP in CHO fibroblasts are mediated through protein kinase. The second class has a normal kinase and is presumed to be blocked in a more distal step of cAMP action.

(2) We have isolated a large number of independent adhesion-defective CHO cells. Two classes of mutants have been analyzed. There is an alteration of a major polypeptide in each as revealed by two-dimensional gel electrophoresis. These major polypeptides have not been previously assigned functions and are presumed on the basis of our work to have a role in maintenance of cell shape or adhesion.

(3) We have devised techniques for quantitatively killing CHO cells using specific cell surface antisera directed against cell surface protein (CSP), myosin, and collagen. These techniques will allow the isolation of mutants lacking surface localization of these antigens.

(4) Toxic derivatives of ligands known to bind to CHO surface receptors have been synthesized and these will be used to isolate mutants lacking these receptors or unable to internalize the ligand-receptor complexes.

Significance for Cancer Research and the Program of the Institute: CHO cells will cause tumors in appropriate hosts. The identification of mutant CHO cells with specific defects in cell surface functions and in regulation of cellular morphology will enable us to determine whether any of these functions are needed for tumor formation. Once this information is obtained, specific therapy aimed at neutralizing those cell surface or cytoskeletal functions required for tumor formation can be devised. Objective 3, Approach 3.5.

Proposed Course: To isolate many classes of mutants with abnormal cell behavior and determine specific protein alterations in these mutants.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08706-07 LMB

PERIOD COVERED

October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

Mammary Carcinogenesis: Mouse Mammary Tumor Virus - Host Cell Interaction

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G.H. Smith	Research Biologist	LMB	NCI
Other:	T.J. Henry	Cancer Specialist	LMB	NCI

COOPERATING UNITS (if any)

LPP, LB, DCBD, NCI
Department of Cell Biology, Baylor University

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Gene Regulation Section

INSTITUTE AND LOCATION

National Cancer Institute, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Isolation of intracytoplasmic A particles from primary and transplanted mouse mammary tumors. Biochemical, immunologic, ultrastructural, and biologic characterization of the purified particles and comparison of those results with those obtained with similarly purified mouse mammary tumor virus (MMTV). A particle proteins were isolated from SDS-polyacrylamide gels, three MMTV antigenic determinants P27, P14, P10 were demonstrated to be associated with A particle structural polypeptides by immunodiffusion. A precursor-product relationship between A particles and MMTV nucleoprotein cores is becoming apparent. Single stranded DNA found bound to A particles in situ is not apparently complementary to viral RNA or to MMTV cDNA (CAP). Studies are in progress to determine the relationship of this DNA to mouse unique and related sequences and to normal cell cytoplasmic DNA(s). Chemical viral co-carcinogenesis in non-MMTV-expressing $C_3H/StWi$ mice is also under study.

PHS-6040
(Rev. 10-76)

Serial No. Z01 CB 08706-07 LMB
1. Laboratory of Molecular Biology
2. Gene Regulation Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
October 1, 1977 through September 30, 1978

Project Title: Mammary Carcinogenesis: Mouse Mammary Tumor Virus - Host Cell Interaction

Principal Investigator: Gilbert H. Smith, Research Biologist, Laboratory of Molecular Biology, DCBD, NCI

Other Investigators: Timothy J. Henry, Cancer Expert

Cooperating Units: LPP, LB, DCBD, NCI
Department of Cell Biology, Baylor University

Man Years: October 1, 1977 through September 30, 1978

Total:	3.0
Professional:	2.0
Other:	1.0

Project Description:

Objectives: The objective of the project is to elucidate the cell-virus interactions in the mouse mammary gland that leads to cellular hyperplasia and eventually to neoplasia. The approach includes attempts to analyze biochemically and biologically the intracellular events occurring during mouse mammary tumor virus infection which accompany preneoplasia and neoplasia. We have initiated investigations into the role of chemical carcinogens in mammary cancer to determine whether they operate synergestically or independent of the virus in the development of cancer in the mouse. To this end we are evaluating a new mouse model for experimental breast cancer, which gives promise of greater relevancy to the human condition. Special emphasis will be given to the physiological state of the epithelial cells in relationship to their response to the carcinogenic agent.

Major Findings: Intracytoplasmic A particles (CAP) were shown to have antigenic determinants for mouse mammary tumor virus (MMTV) structural polypeptides, P27, P14, and P10. Antibody was raised against the major structural polypeptide of CAP (P82). This antiserum precipitated MMTV P27, P23, P14 and P10 in radioimmune precipitation tests with I^{125}-labeled MMTV. These MMTV polypeptides are known to be structural proteins of the virus nucleoprotein core. A particle proteins can be processed by the proteolytic action of trypsin, chymotrypsin or papain to MMTV-like polypeptides MMTV P27 and P10 which possess the respective MMTV-specific antigenic determinants. A manuscript describing these results has been accepted for publication.

Further characterization of the single-stranded DNA found tightly associated with intracytoplasmic A particles has been completed. Hybridizations showed that the DNA was not related to either MMTV (type B virus) to MuLV (Rauscher; type C) by direct hybridization to their genomic RNAs or by competition for sequences complementary to the viral RNAs in the mouse genome. CAP SS DNA hybridized to mouse DNA from various tissues both neoplastic and normal. The extent of hybridization at low driver ratios and the rate of reassociation suggests CAP DNA is a set of sequences whose total complexity is large. Nevertheless SS CAP DNA does not reassociate to itself or with separate isolates of CAP SS DNA. Therefore it does not appear to be a simple contamination with cell DNA. It's relationship to other cytoplasmic DNA fractions is being studied to solve this conundrum.

Studies with Dr. Barbara Vonderhaar, LPP, DCBD, have demonstrated that virgin mouse mammary epithelial cells are unable to make specific milk proteins, alpha-lactalbumin and casein, in the presence of insulin, hydrocortisone and prolactin when their DNA synthesis is inhibited by cytosine arabinoside or 5-fluorouridine. However, they are able to cytologically differentiate and to take on the ultrastructural appearance of secretory cells. Further investigation with the help of Dr. Jeffery Rosen, Baylor University, has shown that casein messenger RNA is made at the same levels in the blocked cells as in unblocked controls. This demonstrated that the block was apparently post-transcriptional possibly post-translational. Primiparous non-pregnant glands were not blocked from synthesizing α-lactalbumin or casein by DNA inhibitors suggesting that one pregnancy satisfies some requirement wherein de Novo DNA synthesis is not required for complete response to the hormonal stimulation for differentiated function. Immunoperoxidase studies have been initiated to quantitatively and qualitatively determine which mammary epithelial cells are capable of synthesizing casein under our experimental conditions in the virgin and whether these cells accumulate immunologically-detectable casein in the blocked glands.

These results will be compared to the appropriately stimulated and blocked primiparous glands. Since these glands are expressing MMTV, we will also investigate the possibility of viral influence on hormonal stimulation in this system. A paper describing some of our preliminary results has been accepted for publication. A more complete report is in preparation.

In an attempt to develop a more relevant experimental mouse model for human breast cancer C_3H/StWi mice, who lost MMTV spontaneously 2 years ago and who do not express endogenous MMTV sequences, were given 7-12 dimethyl benzanthracine (DMBA) a potent chemical carcinogen. These studies were carried out in collaboration with Drs. Daniel Medina and Susan Socher, Department of Cell Biology, Baylor University. We found C_3H/StWi virgin females to be highly susceptible to DMBA, developing mammary tumors (17/37) within six months of receiving the drug. The tumors were mainly papillary adenocarcinomas which arose from intraductal hyperplasias. No alveolar hyperplasias, commonly associated with MMTV were detected. Evaluation of the tumors for the presence of induced MMTV by electron microscopy and molecular hybridization suggested

that expression of MMTV proviral sequences may not be involved in these neoplasms. A manuscript describing this study has been submitted for publication. Further development of this mouse mammary cancer system is underway, e.g. $C_3H/StWi$ virgins, exogenously infected with MMTV, are being tested for their response to DMBA and immunologic studies are underway to test both non-virus and virus-expressing mice for expression of proviral genes.

Our earlier results demonstrating the conversion of intracytoplasmic A particle structural proteins to MMTV-like polypeptides with concomitant preservation of MMTV P27 antigenic determinants has been confirmed and extended in this laboratory and in others. All three internal, core-related MMTV antigenic determinants have been demonstrated to be present in intracytoplasmic A particles. This fact, coupled with the requirement of A particle morphologic integrity for proper precursor-processing has developed a strong argument that A particles represent intracellular MMTV nucleocapsid structures comprised of incompletely processed MMTV structural proteins.

A mouse model with a potential as more relevant to human mammary cancer appears to be the $C_3H/StWi$ mouse subline. These mice develop preneoplastic dysplasias and neoplasms whose histologic nature is similar to the human disease in the absence of overt expression of the endogenous MMTV sequences when challenged with chemical carcinogens. Further studies on these mice give promise toward clarification of the interaction between viral, chemical and hormonal influences in mammary carcinogenesis.

Significance for Cancer Research and the Program of the Institute: National Cancer Plan Objective 3, Approach 3. The mouse mammary tumor virus problem has been and is being used as a relevant model for understanding human mammary neoplasia. Yet our knowledge of the intracellular events in MTV infection is essentially nil. The project is designed to provide a base to further our understanding of the intracellular events with the aim of elucidating the replicative cycle of MTV. Subsequently, it is hoped, armed with this information, to specify the tumorigenic influence of the virus.

Proposed Course: To try to specify the tumorigenic influence of the mammary tumor virus, its relationship to normal differentiation and to the cellular response to chemical carcinogenesis.

Publications:

Michalides, R., Nusse, R., Smith, G.H., Zotter, S.T. and Muller, M.: Relationship between nucleic acids associated with intracytoplasmic A particles and mouse mammary tumor virus RNA. Gen. Virol. 37: 511-521, 1977.

Cardiff, R.D., Puentes, M.D., Young, L.J.T., Smith, G.H., Teramoto, Y.A., Altrock, B.W. and Pratt, T.S.: Serological and biochemical characterization of the Mouse Mammary Tumor Virus with Localization of P10. Virology 85: 157-167, 1978.

Evans, W.H., Mage, M.C., Hsu, C.K., Himmelhoch, S.R. and Smith, G.H.: Transplantable granulocytic leukemia in strain 13 guinea pigs. Cancer Res. 38: 130-136, 1978.

Vonderhaar, B.K., Smith, G.H., Pauley, R.J., Rosen, J.M. and Topper, Y.J.: A difference between mammary epithelial cells from mature virgin and primiparous mice. Cancer Res. 1978, in press.

Smith, G.H.: Evidence for a precursor-product relationship between intracytoplasmic A particles and mouse mammary tumor virus cores. J. Gen. Virol. 1978, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08707-05 LMB

PERIOD COVERED

October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

The Relationship of Genetic and Non-Genetic Factors in Mouse Mammary Tumorigenesis.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. Vlahakis	Research Biologist	LMB NCI
Other:	H.C. Chopra	Research Biologist	LTVG NCI

COOPERATING UNITS (if any)

LTVG, NCI

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Gene Regulation Section

INSTITUTE AND LOCATION

National Cancer Institute, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.75	0.5	0.25

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Investigations continue into the relationship of genetic and non-genetic factors in mouse mammary tumorigenesis. Interest is in the different lines of naturally occurring mammary tumor virus (MTV), and the genetically variable inbred mouse strains. Work is also concerned with the manner of transmission of the various MTV's, i.e. whether by female or equally well by either parent. Finally, strains of mice that develop hormone responsive mammary gland tumors and hyperplastic alveolar nodules (HAN), from which malignant mammary tumors arise are also being studied.

PHS-6040
(Rev. 10-76)

Serial No. ZO1 CB 08707-05 LMB
1. Laboratory of Molecular Biology
2. Gene Regulation Section
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
October 1, 1977 to September 30, 1978

Previous Serial Number: ZO1 CB 08707-04

Principal Investigator: George Vlahakis, Research Biologist, Laboratory of Molecular Biology, DCBD, NCI

Other Investigators: H.C. Chopra, Research Biologist, LTVG, NCI

Cooperating Units: LTVG, NCI

Man Years: October 1, 1977 to September 30, 1978

Total:	0.75
Professional:	0.5
Other:	0.25

Project Description:

Objectives: To compare the naturally occurring mammary tumor virus (MTV) from the high European mammary tumor strain GR, with MTV's from tumor strains C3H, A and DD.
Results: This study is complete. JNCI (59: 447-449, 1977). The GR-MTV proved to be less effective than C3H- and A-MTV's, but similar to DD-MTV in the production of tumors of the mammary gland.

Objectives: To determine whether or not strain DD males have the ability to transmit MTV as in the similar and possibly related strain GR.
Results: This study is complete. JNCI (59: 1553-1555, 1977). There was no evidence of male transmission of MTV in strain DD when outcrossed to MTV-negative strains C57BL and BALB/c. The mode of vertical transmission of MTV in high tumor strain DD is by milk, whereas in strain GR transmission of MTV is accomplished by either parent and therefore is unique among mammary tumor mouse strains currently in use in cancer biology.

Objectives: To determine whether or not MTV-negative substrain C57BL/Haag female mice are susceptible to the development of mammary tumors when given low oncogenic milk-borne MTV from strain DD.
Results: This study is complete and has been accepted for publication in the International Journal of Cancer. C57BL/Haag becomes a line with a high incidence of mammary tumors occurring in the females at a late age. Since both albino strains DD and RIII originated in Europe, develop pregnancy-dependent mammary tumors, carry black and agouti genes (cc, BB, AA), and therefore they may be related, evidence is presented which suggests that the two naturally occurring MTV's from DD and RIII may also be related, and that they may share a common origin.

Collaborative studies with other investigators both here and outside the NIH are continuing again this year. Our contribution of valuable mammary tumor and hepatoma mouse strains, and our knowledge of the biology of these neoplasms make such studies desirable, and therefore are being pursued. One mammary tumor study with Dr. Y.N. Sinha of the Scripps Clinic and Research Foundation, La Jolla, California, has now been published in the Journal of Endocrinology, and a second manuscript is being prepared for later this year.

We are planning to publish a paper with Dr. Melvin Reuber of the Frederick Cancer Research Center on the spontaneous uterine tumors arising in our female mice. This publication should prove useful to those investigators who are working with mice and possibly other rodents.

Some time has also been spent in discussions with other research workers, helping them to develop their own programs. Again, this year, as has been the custom, student groups have been invited into the laboratory for demonstrations and lectures as part of their learning experience.

Significance to Cancer Research and the Program of the Institute: The spontaneous occurrence of mammary tumors in mouse strains depends on the interplay of several factors: host genotype, mammary tumor virus (MTV), hormones, environment. Studies of mammary tumorigenesis in mice need to be continued so that eventually mechanisms of interaction between the various factors will be understood and therefore, quite possible, provide insight into the disease process in man.

Proposed Course: The mammary tumor experiments transferred from the Laboratory of Biology to the Laboratory of Molecular Biology are complete. Two studies have been published and a third has been accepted for publication.

Publications:

Vlahakis, G.: Mouse strain GR: A comparative study of its naturally occurring mammary tumor virus. J. Natl. Cancer Inst. 59: 447-449, 1977.

Vlahakis, G., Heston, W.E., and Chopra, H.C.: Transmission of Mammary Tumor Virus in Mouse strain DD: Further Support for the uniqueness of strain GR. J. Natl. Cancer Inst. 59: 1553-1555, 1977.

Schlom, J., Colcher, D., Drohan, W., Kimball, P., Michalides, R., Schochetman, G., and Vlahakis, G.: Propagation and analysis of mouse mammary tumor viruses from different mouse strains. In Microbiology--1977 (Schlessinger, D., ed.). Washington, D.C., American Society for Microbiology, pp 548-551.

Schlom, J., Colcher, D., Drohan, W., Kettmann, R., Michalides, R., Vlahakis, G., and Young, J.: Differences in Mouse Mammary Tumor Viruses. Cancer 39: 2727-2733, 1977.

Sinha, Y.N., Salocks, C.B., Vanderlaan, W.P., and Vlahakis, G.: Evidence for an Influence of Mammary Tumor Virus on Prolactin Secretion in the Mouse. J. Endocr. 74: 383-392, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08709-03 LMB

PERIOD COVERED
October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

Relationship of Carbohydrate and NAD^+ Metabolism to Cell Growth

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	George S. Johnson, Research Chemist	LMB NCI
Others:	Venkat R. Mukku, Guest Workter	LMB NCI
	Carolyn R. Adler, Guest Worker (summer student)	LMB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, National Institutes of Health, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Escherichia coli growth is quickly inhibited by picolinic acid or 1,10-phenanthroline. Growth resumes after a short adaptation period. Inhibition is overcome by Mn^{++} and can be attributed to an inhibition of RNA synthesis. Guanosine 5'-diphosphate 3'-diphosphate increases after this treatment in some but not all Escherichia coli cell lines.

Cultured mammalian NRK cells treated with picolinic acid or deprived of isoleucine are very responsive to prostaglandin E_1. The activity of adenylate cyclase in membranes from these treated cells is increased.

NRK cells treated with inhibitors of IMP dehydrogenase, to lower GTP levels, lose 50-80% of the response to isoproterenol or prostaglanding E_1.

PHS-6040
(Rev. 10-76)

Serial No. ZO1 CB 08709-03 LMB
1. Laboratory of Molecular Biology
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
October 1, 1977 through September 30, 1978

Project Title: Relationship of Carbohydrate and NAD^+ Metabolism to Cell Growth.

Previous Serial Number: XO1 CBD 8209-02 LMBGY

Principal Investigator: George S. Johnson

Other Investigators: Venkat R. Mukku
Carolyn R. Adler

Cooperating Units: None

Man Years: October 1, 1977 through September 30, 1978

Total:	2.3
Professional:	2.0
Others:	0.3 (summer help)

Project Description:

Objectives: The addition of the pyridine derivative, picolinic acid, to the medium of cultured mammalian cells has pronounced effects on cell growth and cyclic AMP metabolism. The object of this project is to delineate the mechanism of action of this pyridine derivative with emphasis on divalent cations and RNA synthesis. Effects in the bacterium, Escherichia coli, will also be tested and compared with effects in mammalian cells.

Methods Employed: Standard culture techniques will be used to grow cells. Thin layer chromatography and fluorescent analysis will be used to analyze the metabolic components.

Major Findings: Effect of Picolinic Acid on Growth of Escherichia coli. Picolinic acid (2-pyridine carboxylic acid) reversibly arrests growth of cultured mammalian cells. Early studies indicated that growth arrest was due to interference with NAD^+ metabolism. To help explain this inhibition, we tested the effect of picolinic acid on growth of the bacterium, Escherichia coli where cells with mutations in NAD^+ metabolism are available. We found that growth of Escherichia coli quickly ceased after addition of picolinic acid and resumed after a short adaptation period. Similiar effects on growth were found with other chelating agents, and also nicotinic acid or nicotinamide did not prevent the inhibition. This suggests that in Escherichia coli picolinic acid acts through chelation of ions essential for growth and not through NAD^+.

The primary event leading to growth inhibition in Escherichia coli is an inhibition of RNA synthesis. RNA polymerase is a Zn^{++}-containing enzyme. Therefore, inhibition could be direct through chelation of

these essential ions. Alternatively, inhibition could be indirect through an alteration in metabolism of molecules such as guanosine 5-diphosphate 3-diphosphate (ppGpp) which regulate RNA synthesis. Picolinic acid increases ppGpp in some, but not all cell lines. Thus ppGpp is not the mediator of RNA synthesis inhibition.

Effect of Picolinic Acid on Cyclic AMP Metabolism in NRK Cells. NRK cells treated with picolinic acid are very responsive to agents which activate adenylate cyclase and thereby increase cyclic AMP levels in the cell. Adenylate cyclase in membranes from picolinic acid treated NRK cells is 2-3 times more responsive to activation by GTP plus prostaglandin E_1 (PGE_1) than is adenylate cyclase in control membranes. Incubation of control membranes with picolinic acid also increases adenylate cyclase activation by GTP plus PGE_1 but less than similiar treatment of intact cells. Chelation of divalent cations may be involved since EGTA treatment of membranes also increases the response to GTP plus PGE_1.

In another series of experiments we found that NRK cells deprived of isoleucine are also very responsive to PGE_1. Since both isoleucine deprivation and picolinic acid treatment are known to arrest cells at a specific point in the cell cycle, the increased cyclic AMP response may be a cell cycle dependent phenomenon.

We also found that cells treated with inhibitors of IMP dehydrogenase, to lower GTP, lose 50-80% of the response to isoproterenol or PGE_1. This study provides evidence in the intact cell that GTP is required for adenylate cyclase activation.

Significance for Cancer Research and the Program of the Institute: By understanding how growth and metabolism are regulated and by understanding differences in this regulation between normal and transformed cells we may learn to control the growth of certain cancer cells.

Proposed Course: Further studies will be done to understand the molecular basis for the growth inhibition by picolinic acid. Regulation of RNA synthesis will be studied in Escherichia coli in more detail. Studies will be done in cultured mammalian cells to determine if picolinic acid arrests growth through inhibition of RNA synthesis in these cells as well as in Escherichia coli.

Additional studies on the activation of adenylate cyclase will be done. Emphasis will be placed on effects of picolinic acid and isoleucine deprivation on GTP.

Publications:

Fernandez-Pol, J.A., Bono,V.A., and Johnson, G.S.: Control of Growth by Picolinic Acid: Differential Response of Normal and Transformed Cells. Proc. Natl. Acad. Sci. USA, 74: 2889-2893, 1977.

Fernandez-Pol, J.A., and Johnson, G.S.: Selective Toxicity Induced by Picolinic Acid in Simian Virus 40-transformed Cells in Tissue Culture. Cancer Res. 37: 4276-4279, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 CB 08710-03 LMB

PERIOD COVERED

October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

DNA Replication In Vitro Catalyzed by E. coli proteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Sue Wickner, Research Chemist LMB NCI

Other: None

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Biochemical Genetics Section

INSTITUTE AND LOCATION

NCI, National Institutes of Health, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPRDPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Several proteins involved in in vitro DNA synthesis have been purified from E. coli. These proteins include those that are genetically identified and required for E. coli chromosome replication, namely, dnaB, dnaC(D), dnaE, dnaG, and dnaZ gene products, and those that are identified by their requirement in reconstituted DNA replication reactions, namely, DNA binding protein, replication factors X, Y, and Z, and DNA elongation factors I and III (DNA EF I and III). DNA synthesis reactions catalyzed by these proteins have been dissected into partial reactions. DNA synthesis with single-stranded phage DNA first requires the synthesis of an oligonucleotide primer for DNA elongation. With fd DNA, this is accomplished by RNA polymerase. With G4 and ST-1 DNA, dnaG protein catalyzes the synthesis of specific oligonucleotides using ADP and dNTPs or rNTPs. With ØX174 DNA, reactions involving dnaB and dnaC(D) proteins, DNA binding protein, and replication factors X, Y, and Z precede oligonucleotide synthesis by dnaG protein. DNA elongation of primer single-stranded DNA requires dnaZ protein, DNA polymerase III, DNA EF I, and DNA EF III. Such interactions of replication proteins with each other, nucleotides, and DNA have been studied.

Serial No. ZO1 CB 08710-03 LMB
1. Laboratory of Molecular Biology
2. Biochemical Genetics Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
October 1, 1977 through September 30, 1978

Project Title: DNA Replication In Vitro Catalyzed by E. Coli Proteins

Previous Serial Number: ZO1 CB 8710-02 LMB

Principal Investigator: Sue Wickner

Other Investigators: None

Cooperating Units: None

Man Years: October 1, 1977 through September 30, 1978

Total:	1.0
Professional:	1.0
Others:	0

Project Description:

Objective: To gain information about basic biochemical mechanisms involved in the process of DNA replication.

Methods Employed: Standard biochemical techniques required for enzyme purification and characterization and for nucleic acid sequencing.

Major Findings:

1. Initiation of G4 and ST-1 phage DNA synthesis.

G4 and ST-1 DNA synthesis have been reconstituted in vitro with purified dnaG protein, DNA binding protein, ADP, 4 dNTPs, and DNA elongation components. The DNA synthesis reaction has been separated into a priming reaction and a DNA elongation reaction. The priming reaction is proceeded by the specific binding of dnaG protein to G4 or ST-1 DNA that is covered with DNA binding protein. The dnaG protein catalyzes the synthesis of a unique oligonucleotide dependent on G4 or ST-1 DNA, ADP, and rNTPs or dNTPs. ADP appears to be incorporated at the 5'end and AMP appears to be incorporated internally. With G4 DNA, the oligonucleotide sequence is: pppAGUAGGGACGGCGGCUUUCGCCGUCCAU, and could have the structure of a stem and a loop (first shown by Bouche, Rowen, and Kornberg). This sequence is complementary to the DNA sequence obtained by N. Godson. With ST-1 DNA the oligonucleotide synthesized by dnaG protein is similar to that synthesized with G4 DNA: pppAG GAGGGAAGGCGGCUAACGCCUUCCACG, and is also conplementary to the DNA sequence obtained by Simms and Dressler. While these oligonucleotides can be about 30 nucleotides long, shorter oligonucleotides can serve as efficient primers for DNA synthesis; the 5' ends are unique and the 3' ends are variable. With other single-stranded

phage DNAs, the dnaG protein is unable to bind to or to catalyze the synthesis of oligonucleotides. Once the primer has been synthesized, DNA elongation can be catalyzed by the combination of DNA polymerase III, dnaZ protein, DNA elongation factors I and III, and dNTPs.

2. Initiation of ØX174 phage DNA synthesis.

ØX174 DNA synthesis has been reconstituted in vitro with purified dnaB, dnaC(D), and dnaG proteins, DNA binding protein, three other E. coli proteins defined only by their requirement in this reaction (referred to as replication factors X, Y, and Z), ATP, dNTPs, and DNA elongation components. Enzymatic activities of some of these proteins have been studied independent of their role in ØX174 DNA synthesis. The dnaB protein is a ribonucleoside triphosphatase that is stimulated by single-stranded DNA, all 4 rNTPs, are hydrolyzed. The dnaC(D) protein inhibits the DNA-independent ATPase activity of dnaB protein and forms a complex with dnaB protein in the presence of ATP. No enzymatic activities have been found associated with dnaC(D) protein and replication factors X and Z. Replication factor Y is a single-stranded DNA-dependent ATPase or dATPase.

The ØX174 DNA synthesizing reaction has been divided into partial reactions by the isolation of various protein-DNA intermediates. DNA binding protein and replication factors Y and Z, bind, without specificity, to any single-stranded DNA; the binding of any one is not dependent upon the other two. While ØX174 DNA synthesis requires high levels of DNA binding protein such that the DNA is completely covered, it requires low levels of replication factors Y and Z, perhaps only one or several molecules per circle of DNA. When DNA binding protein, replication factors X, Y, and Z, dnaB and dnaC(D) proteins, ATP, and ØX174 DNA are incubated together, dnaB protein is transferred from a protein complex with dnaC(D) protein to the protein-DNA complex containing DNA binding protein and replication factors Y and Z. This protein-DNA complex contains dnaB protein, DNA binding protein, and replication factors Y and Z; it does not contain dnaC(D) protein or factor X. DNA synthesis by this isolated complex requires dnaG protein, dNTPs, and DNA elongation components. It does not require, a second addition of dnaC(D) protein or replication factor X. Presumably dnaG protein catalyzes the synthesis of a primer for DNA elongation by recognizing a site on the ØX174 DNA created by the action of the proteins required for the prepriming reaction. This complex mechanism by which ØX174 DNA is primed is template specific; with identical reaction conditions, dnaB protein cannot be transferred to other single-stranded circular DNA.

Significance for Cancer Research and the Program of the Institute: The central process of heredity and cell growth is the replication of the genetic material. Studies of this process in E. coli have been made possible by the availability of (1) mutants defective in DNA synthesis and (2) large amount of bacteria necessary for biochemical studies. It is expected that the understanding that this work is generating about the process of DNA replication in E. coli will shed light on the nature of the same process in animal cells. Perhaps if normal replication were understood, we would be better able to study the abnormalities of cancer cells.

Proposed course: To continue studying biochemical mechanisms involved in DNA synthesis using purified proteins and defined single-stranded DNA templates. Also, to study unique questions of double-stranded DNA replication by first developing an in vitro DNA synthesizing reaction dependent on double-stranded λdv plasmid DNA. These questions include: (1) how is double-stranded DNA replication initiated? (2) how are the two parental strands separated during chain propagation? (3) how is the chain which grows in the overall 3' to 5' direction synthesized? (4) how is replication terminated and the two daughter molecules separated.

Publications:

Wickner, S.: DNA or RNA priming of G4 DNA synthesis by E. coli dnaG protein(1977). Proc. Natl. Acad. Sci. USA 74: 2815-2819.

Wickner, S.: DNA replication proteins of E. coli (1978). Ann. Rev. Biochem , in press.

Wickner, S.: G4 and ST-1 DNA synthesis in vitro (1978). In DNA synthesis: Present and Future ed. I. Molineux, M. Kohiyams, New York: Plenum (1978) in press.

Wickner, S.: Conversion of single-stranded phage DNA to Duplex DNA in vitro (1978). In The Single-stranded phages ed. D. Denhardt, D. Dressler, D. Ray, New York: Cold Spring Harbor Laboratory, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08711-02 LMB

PERIOD COVERED

October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

Mechanism of Site-Specific Recombination in Bacteriophage Lambda.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Susan Gottesman, Research Chemist	LMB NCI
	Max Gottesman, Head, Biochemical Genetics Section	LMB NCI
Other:	Patsy Trisler, Research Biologist	LMB NCI
	Kenneth Abremski, Guest Worker	LMB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Biochemical Genetics Section

INSTITUTE AND LOCATION

NCI, National Institutes of Health, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have been studying the biochemistry and genetics of lambda site-specific recombination. An E. coli mutation, rap, which blocks the growth of normal lambda is permissive for lambda variants with deletions at or near the attachment site. We are improving our in vitro site-specific recombination assay with a view to isolating and purifying the components.

PHS-6040
(Rev. 10-76)

Serial No. ZO1 CB 08711-02 LMB
1. Laboratory of Molecular Biology
2. Biochemical Genetics Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
October 1, 1977 through September 30, 1978

Project Title: Mechanism of Site-Specific Recombination in Bacteriophage Lambda.

Previous Serial Number: ZO1 CB 8711-01

Principal Investigators: Susan Gottesman, Max Gottesman

Other Investigators: Patsy Trisler
Kenneth Abremski

Cooperating Units: None

Man Years: October 1, 1977 through September 30, 1978

Total:	2.5
Professional:	2.5
Other:	0

Project Description:

Objectives: Bacteriophage lambda inserts into and excises from the E. coli chromsome. This recombination occurs at a unique site in the viral and host chromosomes, and is promoted by several functions. We have previously demonstrated the excision reaction in vitro. Our object is to continue to study the mechanism of phage excision, by purifying and analyzing the components of the in vitro system.

An E. coli mutant, rap, is non-permissive for wild-type lambda, but plates lambda variants with altered attachment sites. We intend to study this phenomenon.

Methods Employed: Standard microbial genetic and biochemical techniques.

Major Findings: (1) The substrate for integrative recombination is lambda DNA in the form of a covalent, negatively-twisted circle. We have been investigating the substrate requirements for the excisive reaction in detail. Coumermycin, an antibiotic which inhibits the supercoiling of DNA in vivo and in vitro, inhibits integrative recombination as well. It does not inhibit the excisive reaction in vitro, and may only inhibit it in vivo under certain conditions. This suggests that the mechanism of recombination in vitro may proceed by a different route for the integrative and excisive recombination. (2) An agarose gel assay system has been developed for the excisive recombination reaction, which allows analysis of the reaction product in the absence of viable phage DNA. Various substrates have been developed which can be used in other assay systems currently

being developed. (3) Int and Xis have been separated from host factors necessary for the excision reaction; Xis purification is commencing. (4) The bacterial mutation rap, which inhibits the growth of phage with normal attachment regions, maps near the his locus of the bacterial chromosome. Mutations in the bacteriophage which allow growth on the rap bacterial strain are most commonly deletions to the left of att, in the b2 region of the phage map. Our experiments indicate that a product coded for by this region leads to abnormal phage development. The loss of the same function may make excision xis independent.

Aims Identification of b2 product and its role in normal phage development; purification of Xis and host factors involved in excision; identification of host factors involved in rap defect.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 CB 08712-03 LMB

PERIOD COVERED

October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

The Role of Plasma Membrane Proteins in the Regulation of Cell Behavior.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ira H. Pastan, Chief, LMB	LMB NCI
	Mark Willingham, Senior Surgeon	LMB NCI
Others:	Robert Shiu, Guest Worker	LMB NCI
	Fred Maxfield, Guest Worker	LMB NCI
	James Keen, Staff Fellow	LMB NCI
	Ellis Neufeld, Physical Science Aid	LMB NCI

COOPERATING UNITS (if any)

Bob Pratt, Dental Research Institute

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, National Institutes of Health, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.4	3.1	.3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Mutant AD6, defective in cell-substratum adhesion has a defect in N-acetylglucosomine acetylase and a decrease in binding of Epidermal Growth Factor. An antibody to GPP78, a protein that accumulates in AD6 and glucose-starved cells, shows that GRP 78 is located in internal membranes, and therefore is unlikely to participate in glucose transport as previously suggested.

PHS-6040
(Rev. 10-76)

Serial No. ZO1 CB 08712-03 LMB
1. Laboratory of Molecular Biology
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
October 1, 1977 through September 30, 1978

Project Title: The Role of Plasma Membrane Proteins in the Regulation of Cell Behavior

Previous Serial Number: ZO1 CBD 08210-02 LMBGY

Principal Investigators: Ira H. Pastan and Mark Willingham

Other Investigators: Robert Shiu, Fred Maxfield, James Keen and Ellis Neufeld

Cooperating Units: National Institute for Dental Research

Man Years: October 1, 1977 through September 30, 1978

Total:	3.4
Professional:	3.1
Other:	3.0

Project Description:

Objectives: To identify the membrane proteins that participate in cell adhesion, cell movement and regulate cell metabolism and to determine if these are altered in malignant transformation.

Methods: Cell culture; preparation and analysis of membrane proteins and their effects on cell behavior; isolation of mutants with defective membrane proteins.

Major Findings: The enzymatic basis of the defect in the adhesion defective mutant AD6 was shown to be in the enzyme N-acetyl glucosamine acetylase. This enzyme could not be detected in extracts of the mutant cells. One consequence of the decreased glycoprotein synthesis in AD6 is a fall in the capacity of the cells to bind and respond to epidermal growth factor indictating that the EGF receptor is probably a glycoprotein.

The 78,000 molecular weight protein (GRP78) has been purified from RSV transformed CEF and a specific antibody obtained. The protein was detected in internal membranes and not in the plasma membrane.

The entry of α_2 macroglobulin into cells has been studied in detail by Mark Willingham and Fred Maxfield who has shown that the protein first binds to diffusely distributed receptors, then patches over coated pits and finally enters the cells in coated vesicles. The protein forming the coat of coated vesicles (clathrin) has been isolated by J. Keen who is investigating its properties.

Significance to Biomedical Research and the Program of the Institute: The invasive properties of tumor cells may be due to altered membrane proteins. Identification and isolation these proteins may suggest new methods of cancer treatment. Objective 3, approach 3.5.

Proposed Course: To isolate additional mutants defective in surface properties. To identify other surface proteins important in the control of cell shape and movement.

Publications

Pouyssegur, J., Shiu, R.P.C., and Pastan, I.: Induction of two transformation-sensitive membrane polypeptides in normal fibroblasts by a block in glycoprotein synthesis or glucose deprivation. Cell 11: 941-947, 1977.

Shiu, R., Pouyssegur, J., and Pastan, I.: Glucose depletion accounts for the induction of two transformation-sensitive membrane proteins in Rous sarcoma virus-transformed chick embryo fibroblasts. Proc. Natl. Acad. Sci. USA 74: 3840-3844, 1977.

Moore, L., and Pastan, I.: Energy-dependent calcium uptake activity in cultured mouse fibroblast microsomes. Regulation of the uptake system by cell density. J. Biol. Chem. 252: 6304-6309, 1977.

Pastan, I., Willingham, M., Anderson, W., and Gallo, M. Localization of serum derived α_2M macroglobulin in cultured cells and decrease after Moloney sarcoma virus transformation. Cell 12: 609-617, 1977.

Pratt, R.M., and Pastan, I. Decreased binding of epidermal growth factor to Balb/c 3T3 mutant cells defective in glycoprotein synthesis. Nature 272: 68-70, 1978.

Willinghma, M.C., and Pastan, I. The visualization of fluorescent proteins in living cells by video intensification microscopy (VIM). Cell 13: 501-507, 1978.

Moore, L., and Pastan, I. Energy-dependent calcium uptake by fibroblast microsomes. In Annals of the New York Academy of Sciences: Calcium transport and cell function, (Scarpa, Antonio and Carafoli, Ernesto, Eds.) Vol. 307, pp. 177-194, 1978. (New York Academy of Sciences).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08714-01 LMB

PERIOD COVERED
October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)
Mode of Action of a Bacterial Function Involved in Cell Growth Control

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Susan Gottesman	LMB NCI
	Patsy Trisler	LMB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Biochemical Genetics Section

INSTITUTE AND LOCATION
NCI, National Institutes of Health, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.8	.8	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The lon mutants of E. coli have pleiotropic effects on cell growth: a) they are defective in the formation of septa between dividing cells and therefore form long filaments, b) they overproduce the mucopolysaccharide coating of cells, possibly by disrupting the control of operons involved in synthesis of mucopolysaccharide, such as the gal operon, c) they are defective in the degradation of abnormal proteins. We are investigating the genetic defect in the lon mutants with the aim of determining the interrelationships of these defects.

PHS-6040
(Rev. 10-76)

Serial No. ZO1 CB 08714-01 LMB
1. Laboratory of Molecular Biology
2. Biochemical Genetics Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
October 1, 1977 through September 30, 1978

Project Title: Mode of Action of a Bacterial Function Involved in Cell Growth Control

Previous Serial Number: New project

Principal Investigators: Susan Gottesman, Patsy Trisler

Other Investigator: None

Cooperating Units: None

Man Years: October 1, 1977 through September 30, 1978

Total:	.8
Professional:	.8
Other:	0

Project Description:

Objectives: Lon mutants are mucoid, UV sensitive, and defective in degradation of abnormal proteins. We are investigating the genetics of these mutations as a basis for future biochemical characterization.

Methods Employed: Standard microbial genetic and biochemical techniques.

Major Findings: (1) We have demonstrated that all mutants isolated as defective in degradation of abnormal proteins map at the previously described lon locus. In addition, the degradation defect extends to some missense proteins, as well as the polypeptide fragments previously studied. (2) We have isolated a series of specialized transducing phage carrying various portions of the lon region; with these phage, we can unambiguously order previously isolated lon mutations. (3) In investigating the effect of lon on control of the gal operon, we have isolated mutations in one of the gal transport systems. These mutations help clarify the pathways for use of galactose as a carbon source under severe catabolite repression.

Significance for Cancer Research and the Program of the Institute: An understanding of the growth control of E. coli may serve as a model for understanding growth control in normal and transformed mammalian cells. Protein degradation is believed to play an important role in the control of mammalian cells; insight into such processes should be gained by further investigation of the process in E. coli.

Proposed Course: Identification of genes controlled by lon and the mode of their control; identification of the biochemical defect in protein degradation, and its relation to the lon product; correlation of lon effects

on the gal operon with other detailed studies of the lon operon.

Publications

Gottesman, S. and Zipser, D: Deg Phenotype of Escherichia coli lon Mutants. Journal of Bacteriology 133, 844-851 (1978).

LABORATORY OF PATHOPHYSIOLOGY
ANNUAL REPORT SUMMARY
October 1, 1977 through September 30, 1978

The research effort of the LPP is focused on the following areas:

1. Pathophysiology of mammary gland and tumors.
2. Events related to cell cycle and cell division, particularly in the mammary gland.
3. Biochemical changes related to the onset and cessation of lymphocyte proliferation.
4. Structure and function of biological membranes.
5. Properties and biological significance of extranuclear DNA.
6. Pathophysiology of cachexia in tumor-bearing hosts.
7. Effects of ionizing radiations on proteins, DNA, and bone marrow cells.

1. Pathophysiology of mammary tissue

One group of projects is concerned with the mechanism of hormone-dependent mammary tumor regression (Dr. Cho-Chung and Dr. Bodwin). Work of the past years indicated that during regression: (a) an extensive endocellular digestion occurred; (b) the tumor used as much energy as during growth; (c) extensive new synthesis and more rapid turnover of lysosomal enzymes occurred; and (d) cytosol proteins became more easily hydrolyzed by lytic enzymes within a few hours after hormonal deprivation of the host. During the development of this line of investigation, it was observed that dibutyryl cyclic AMP (DBcAMP) injected into tumor-bearing hosts produced growth arrest and/or regression of several mammary tumors, both hormone-dependent and -independent. Work was concentrated on Walker 256 and DMBA mammary carcinomas. The following results were obtained: (a) Walker 256 tumors regressed under DBcAMP treatment only when endowed with high levels of cAMP binding proteins. (b) Cyclic AMP-binding protein in the hormone-unresponsive Walker 256 as well as in the hormone-dependent DMBA mammary tumors contained the regulatory subunit of protein kinase, type II. (c) The cAMP+receptor complex translocated from the cytoplasm into the nuclei in cAMP-responsive tumors. In cAMP-unresponsive Walker 256, translocation did not occur; however, the isolated nuclei of unresponsive Walker 256 were able to accept the cAMP+receptor complex from the cytosol of the cAMP-responsive Walker 256 carcinomas. This suggests that lack of regression depends on an alteration of the cytoplasmic receptor. (d) Penetration of cAMP+receptor complex into the nucleus results in phosphorylation of a nonhistone protein, ∿76000 MW. (e) Regression following ovariectomy or DBcAMP injection triggers 3 events that are common to both treatments: a decrease of estrogen receptors, an increase of cAMP receptors, and the phosphorylation of an endogenous nuclear protein ∿76000 MW. The reverse relationship between estrogen and cAMP receptor levels in hormone-dependent tumors may have immediate clinical application in the sense that the ratio estrogen:cAMP receptors could be a marker of hormone responsiveness of a mammary carcinoma. Work in this area is being pursued.

Another aspect of mammary gland and tumor pathophysiology is being studied by Dr. Qasba and coworkers, using the rat as experimental model. The major objective of this group of projects is to understand the regulatory mechanism of milk protein production in normal and neoplastic mammary epithelium. Work of previous years has shown that (a) two species of α-lactalbumins could be isolated from rat milk (α-LA_1, αLA_2, and (b) primary mammary carcinomas produced by DMBA or NMU were able to synthesize α-lactalbumin and casein but most transplantable mammary carcinomas could not. In the past year the preliminary work on mammary carcinomas was completed and showed that regulation of α-lactalbumin production under pituitary stimulation appears to vary from one type of mammary carcinoma to another. Characterization of αLA_1 and αLA_2 was pursued. αLA_1 appears to be a unique α-lactalbumin in that: (a) it is a glycoprotein; (b) it has a MW of 21000, larger than other known α-lactalbumins; (c) it is still active in the lactose synthetase assay; (d) it shares a common C terminal with LA_2 but differs in the N terminal amino acids, (e) the peptide map after chymotryptic digestion shows that αLA_1 contains all peptides present in αLA_2 plus 5 peptides; and (f) αLA_1 can be converted to αLA_2 by autodigestion. Purification of rat casein was completed and a RIA was set-up. mRNA for αLA and casein were isolated and purified and their cDNA probes were synthesized. Using these tools the following observations were made: (a) the content of αLA and casein in the mammary gland increased during the 2nd to 5th days of gestation, then declined sharply at the 7th to 9th days of gestation and rose again until parturition. (b) The relative proportion of casein species changes at parturition as compared with gestation, with a 12-fold increment of 25K casein, 2-fold for 45K and practically no change for 29K. (c) mRNA for αLA and casein translated by a wheat germ system showed little activity up to the 7th day of gestation then showed two peaks on the 12th and 17th day. In the lactating gland, αLA mRNA activity increased up to the 4th day, stabilized up to the 14th day and declined thereafter. Casein mRNA activity increased up to the 8th day of lactation, plateaued up to the 14th day and declined thereafter. Other proteins are also translated and follow similar trends. (d) In 3-5 day lactating gland αLA makes up 0.6% and caseins 7.5% of the total protein, giving a ratio of about 0.1:1. From total cellular RNA, αLA mRNA represents 30% of total mRNA activity and casein mRNA the remaining 70%. The ratios of mRNA activities versus total αLA and casein content of the gland do not match. In milk, αLA represents 4.5% and casein 52% of total protein.

The mechanism by which milk proteins are produced and the changes that occur during neoplastic transformation are also being studied by Dr. Vonderhaar and coworkers. Organ cultures of mouse mammary glands are mostly utilized as an experimental model; and attention is focused on the role of thyroid hormones in the functional differentiation of the gland. Previous findings indicated that organ cultures of adult virgin gland in the presence of insulin, prolactin, and hydrocortisone produced casein and αLA after a round of cell division occurred. Present work showed that DNA synthesis *in vitro* is not necessary if the mammary gland of virgins is stimulated *in vivo* by thyroxine before explanting. This was true even when the explant was obtained several weeks after thyroxine treatment when the mammary gland appeared "dormant". Primary cultures of mammary epithelium obtained by collagenase treatment showed morphological signs of secretion when cultivated in the presence of insulin, hydrocortisone and prolactin but no enzymatically active αLA or renin Ca^{++} precipitable caseins were detectable. However, the same system,

prevented from undergoing DNA synthesis by addition of cytosine arabinoside, showed a 4-fold increase in casein mRNA which was translated at about one-fourth of the efficiency of the unblocked system (evaluation by immunoprecipitation of casein peptides). Addition of T3 to the system increased to about 30% the level of casein peptides present. Preliminary data suggest that something similar occurs for αLA. In the attempt to understand how thyroid hormones exert their influence on the mammary gland, specific binding proteins for LT3 were sought and found in both nuclei and cytoplasms of epithelium from euthyroid virgin and midpregnant glands. The cytosol receptor has binding affinity for LT3 ($Kd \sim 10^7\ M^{-1}$) when prepared from fat pad + gland homogenates but also high affinity ($Kd \sim 10^6\ M^{-1}$) prepared from fat pad deprived of epithelium. The properties of cytosol receptors and the role of the fat pad as a possible storage site for thyroid hormones are being further investigated. Meanwhile, two promising observations have been made: (a) addition of T3 to organ cultures of euthyroid mature glands induced prolactin receptors, and a 50% increase in prolactin binding was observed when T3 was added to crude membranes prepared from lactating glands, but not for liver membranes. (b) Like thyroid hormones, prostaglandin F2α overcame the need for DNA synthesis when milk proteins were induced in vitro and in particular, enhanced αLA production. PgF2α also mimicked T3 in overcoming progesterone inhibition of αLA synthesis in pregnant mouse mammary tissue in vitro. The interaction between T3 and PgF2α on mammary epithelium is being further investigated.

Expanding on the hypothesis that high risk of neoplastic transformation may be revealed in mammary epithelium by an increased angiogenic capacity, Dr. Maiorana completed experiments showing that the hypothesis is also valid for rat mammary gland. In the attempt to counteract progression of high risk mammary epithelium toward neoplastic transformation, Dr. Maiorana is also completing work on the role that vitamin A derivatives may have in preventing mammary tumor progression in C3H-A^{vy} mice. Preliminary data indicate that retinyl acetate has negligible preventive capacity. Evaluation of other compounds is under investigation.

Dr. Jain continued to expand the study of parameters concerned with heat generation and thermal conductivity of neoplastic tissues. By direct measurement in vivo it was established that under physiological conditions thermal conductivity of Walker carcinoma is 2.8 ± 0.8 MW/cm/°C. When the blood supply to the tumor was doubled or reduced to one-half, the thermal conductivity varied proportionally to the square root of the perfusion rate. Experiments aimed at measuring variations in local perfusion of neoplastic tissue and the efficiency of blood flow restoration after changes in blood temperature are progressing, but final results are not yet available. The final objective of this work is to define basic parameters of heat generation in tumors as an aid to diagnostic thermography.

2. Regulation of cell cycle and cell division

The study of poly(ADP-Ribose) and its role in the initiation of cell division has been continued by Drs. Stone and Kidwell. Attention has been concentrated on the interaction between poly(ADP-Ribose) and basic chromosomal proteins, histone H1 in particular, to which the polymer is associated. The following observations were made: (a) Chromatin-bound poly(ADP-Ribose)

polymerase attaches polymer chains to histone H1 producing a complex composed of two H1 molecules cross-linked by a single polymer chain. (b) The polymer is in a protected environment within the complex. (c) The H1+polymer complex binds to DNA and exposes the polymer, and (d) conformational changes of chromatin produced by polyamines selectively promote H1+poly(ADP-Ribose) complex formation. The hypothesis that poly(ADP-Ribose) serves as a bridge between proximal folds of the chromosomal fiber thus determines modification of chromatin structure. These modifications are believed to be essential in the progression of cell division since arrest of poly(ADP-Ribose) synthesis produces cell death.

Drs. Kidwell and Wicha have expanded the analysis of conditions favoring growth of rat mammary epithelium and are concentrating their attention on the interaction between epithelium and the adipocytes of the fat pad. A test system was developed whereby fragments of ducts and alveoli were isolated from the adipocytes but still consist of mammary epithelium surrounded by myoepithelium, type IV collagen and glycoproteins of the basal membrane. These organoid structures kept in an appropriate culture medium produced αLA and a halo of proliferating cells. Saturated fatty acids inhibited cell growth while unsaturated fatty acids favored growth. To this end, each fatty acid had its optimal concentration that correlated with the relative concentration of fatty acids in the gland. Oleic acid favored growth of neoplastic epithelium while linoleic and linolenic acids stimulated growth of normal epithelium.

Dr. Knazek and coworkers continued their work on prolactin receptors. They had previously shown that: (a) the Snell dwarf mouse lacks prolactin receptor in the liver, (b) ovine prolactin or bovine growth hormones induced them, and (c) phospholipase A_2 enhanced prolactin binding. In the past year they established that: (a) growth hormone but not the prolactin contaminant, was indeed able to induce prolactin receptors, and (b) prolactin receptor induction was not prevented by cycloheximide. On the assumption that the effect of phospholipase A on prolactin receptors was indicative of a participation of fatty acids in the induction of the receptors, mice were fed a diet lacking the essential fatty acids. Prolactin receptor level decreased about 30% and prolactin was unable to enhance this level. Injections of indomethacin produced the same effects. Photofluorimetric polarimetry of membrane preparations failed thus far to reveal changes in their microviscosity. Thus, it is not certain that the effects of the deficient diet on the prolactin receptors depends on a modification of membrane composition.

In collaboration with the Department of Chemical Engineering, University of Delaware, the artificial capillary culture technique has been further improved by developing a culture unit containing two separated capillary bundles braided together. Conditions were developed to permit one bundle to be simultaneously operated at a higher pressure than the other. This permitted an evaluation of the conditions controlling the transfer of labeled compounds of various molecular weights through solid cell masses grown between the capillaries; it also served to establish the role of convection and diffusion modes of mass transport within solid tumors.

3. Biochemical changes related to onset and arrest of growth in lymphocytes.

In previous work a detailed analysis of ribosomal subunit participation

in initiating translation was carried out in resting and growing human lymphocytes. (Dr. Cooper and co-workers). In the past year the study progressed, and actinomycin D was used as a probe to demonstrate that in lymphocytes the drug produced a decrease in protein synthesis by action on the nucleus. The kinetics of the actinomycin effect showed that resting lymphocytes lost the ability to synthesize proteins gradually but very rapidly lost the ability to be stimulated by mitogens. These observations suggested that some form of RNA could be the rate limiting component of protein synthesis by resting lymphocytes. Support for the hypothesis was found in 3 observations: (a) in resting lymphocytes Act. D produced an immediate decline of the rate of protein synthesis; (b) in phytohemagglutinin-stimulated lymphocytes a delay of 50 min. was observed between Act. D addition and decline of protein synthesis; and (c) in lymphocytes growing for 24-40 hrs the delaying effect of Act.D was no longer observed. The nature of the RNA with a putative rate limiting function on protein synthesis in resting lymphocytes is being investigated further. Three other projects are ongoing but the results are not yet completed. One concerns the development of a radioisotope competition assay for the Dissociation Factor which controls the mobilization of inactive free ribsomes into polysomes. The second concerns the role of microtubules in lymphocyte activation, and the third relates to the significance of transglutaminase activation observed after mitogen treatment of resting lymphocytes.

Dr. Berger and coworkers succeeded in synthesizing a powerful ribonuclease inhibitor that protects ribonucleoproteins during cell lysis and purification. With the help of the inhibitor it was possible to show that: (a) labile mRNA$^+$-poly(A) was not an artifact due to degradation of hnRNA, (b) hnRNA does not leak out of the nucleus, and (c) labile mRNA$^+$poly(A) must be construed as a legitimate component of the cytoplasm with both a poly(A) tail and a size distribution similar to that of bulk mRNA$^+$poly(A). Accumulation curves were generated in order to determine whether labile mRNA$^+$poly(A) was a precursor of polysome bound mRNA$^+$poly(A). The following conclusions were reached: (a) there was no precursor product relationship between free and bound molecules, and (b) there was no storage of subclasses of free mRNA$^+$poly(A). Investigation of the function of labile mRNA$^+$poly(A) in which amount and half-life were determined in resting and PHA-stimulated lymphocytes, suggested that labile mRNA$^+$poly(A) in the resting cells is probably an overproduction of mRNA which is corrected after growth stimulation.

4. Structure and function of biological membranes

Analysis of milk fat excretion by the freeze-fracture technique showed that most fat droplets are extruded with a bilayer membrane envelope, cleared of membrane particles. Fat droplet formation results by apposition of endoplasmic reticulum cisternae and the process is similar in normal and neoplastic cells of rat mammary gland. The membrane coat of the fat globule in rat milk is in a dynamic state and the joining together of membrane edges occurs in a zipper-like fashion. The process of membrane fusion was further studied utilizing the interaction of Sendai virus with human erythrocytes. The virus binds to the glycophorin molecules of the erythrocyte membrane and the fusion starts with the formation at several points of plasmadesmata which provide continuity between the 2 cytoplasms. From these initial points a solid expansion of the plasmadesmata occurs and a network of membrane tubules with a lumen

continuous with the external space becomes evident. An expansion of the net-work completes the incorporation of the viral into the erythrocyte membrane. Three other model systems were utilized to study the fusion of cell membranes, zoospores of Pythophtora palmivora, human platelets during formation of the fibrin net-work, and the unmyelinated axons of the garfish olfactory nerve at the site of intercellular communication between the axon and the Schwann cell. Finally condensation of vesicles and formation of secondary lysosomes in the rat aspects of secretory events, membrane fusion in particular.

5. Properties and biological significance of extranuclear DNA

The effort to differentiate various types of DNA present in the cytoplasm continued and required a large amount of work and produced a long list of failures. A solution to the problem, however, was found by using CTAB to precipitate the DNA from tissue samples. This method can also separate DNA from most other tissue components and eliminates the requirement of phenol extraction. By careful control of procedures the tissue disruption and subsequent fractionations plus the application of the CTAB-DABA method, it was established that in the rat liver, DNA content of mitochondria is about 0.3% and that of microsomes about 3% of the total liver DNA. These figures are considerably different from previous estimates, indicating that microsomes and mitochondria each contained about 1% of the total liver DNA.

The isolation of melanin granules from B16 melanoma is being pursued by Dr. Liacouras, and the purification of the DNA of these granules is being carried out with the objective of comparing by hybridization the melanin granule DNA with nuclear and mitochondrial DNA.

As a preliminary approach to study the role of microsomal DNA, Dr. Chakrabartty has isolated and partially purified serum albumin-mRNA from rat liver, normal or thioacetamide-treated. Complementary DNA has been synthesized using a partially purified Alb-mRNA template. An analysis is now progressing of: (a) the quantities of RNA poly(A) and Alb-mRNA produced by livers, normal or thioacetamide-treated, and (b) the molecular hybridization of rat unique DNA with RNA from control or treated livers to assess the validity of the hypothesis that microsomal DNA may be a "messenger" DNA.

Dr. Otani is doggedly pursuing his long search for amino acid derivatives that could inhibit growth by acting as antimetabolites. ß-hydroxynorvaline has been prepared and isolated in good yield to be tested first for its effect on microorganisms. The preparation of 18 N-trifluoroacetyl derivatives of amino acids has been completed and 5 have been found with distinct antimetabolic effects in the Lactobacillus casei test. Twenty-three acyl amino acids containing highly lipophylic sidechains and 15 N-benzoil derivatives of amino acids and amino acid analogs have been prepared and are now being purified for final testing. A commercial preparation of N-chloroacetyl-L-tyrosine has been found to have an exceptionally strong inhibitory effect in the L. casei test. The identification of the active principle is being pursued.

6. Pathophysiology of cachexia in tumor bearing hosts

Work carried on in the past by Dr. Morrison supported the hypothesis

that in tumor-bearing rats cachectic hypophagia developed as a consequence of successive deletion of components of the feeding control system until a catastrophic collapse occurred. The cachectic process starts early in tumor development but a behavioral adaptation develops to compensate for a decline in the amount of feeding activity with an increase of feeding efficiency. A set of experiments is now progressing that involves 3 areas: (a) influence on cachexia by interference with individual components of the feeding control, (b) secretion of N by host and tumor during amino acid imbalance or deficiency, and (c) metabolic efficacy of total parenteral nutrition during tumor growth. Results are still incomplete.

7. Effects of ionizing radiations on proteins, DNA and bone marrow cells

Dr. Riesz and coworkers continued their work on the effect of ionizing radiations on DNA and proteins as it relates to loss of their biological activities. The reactions of hydrated electrons (e^-_{aq}) with 55 dipeptides, 18 tripeptides and 25 acetyl and formyl amino acids have been studied by ESR and spin trapping techniques. e^-_{aq} were generated by γ-radiolysis of deaerated aqueous solutions; Na-formate or t-BuOH were added to scavenge the OH radicals, and t-nitrosobutane was employed as the spin trapping reagent. For most of the dipeptides the radical $-CO^--NH^-$ converts to the primary deamination radical CHR'-CONH-CHR-COO^- where R and R' are the side chains of the common amino acids. Only secondary deamination reactions were observed for dipeptides containing β-alanine as the amino terminal residue and for acetyl and formyl amino acids. Addition of e^-_{aq} to tripeptides produced scission of the C-N bond at 3 sites, between the N of the ammonium group and the α carbon and between the N of the peptide linkage and the adjoining α carbons. The radicals corresponding to each of these 3 types of scission were identified. Following the basic approach of producing free radicals by UV photolysis, trapping by t-nitrosobutane and identifying them by ESR spectroscopy, Dr. Riesz and co-workers also studied and reported on: (a) the reactions of the SO_4^- radical with protein and nucleic acid constituents, (b) the radicals produced by reactions of OH radicals with alkyl substituted ureas and amides in aqueous solutions, (c) the reactions of OH radicals with methyl and ethyl derivatives of uracil, cytosine and thymine in aqueous solutions, (d) the reactions of OH radicals with several dihydropyrimidine base derivatives, and (e) the post-irradiation growth of the spin-adduct nitroxide radical produced by the addition of the thymine-OD radical to t-nitrosobutane in γ-irradiated, deaerated D_2O solutions. The final objective of this work is to understand the nature of radiobiological death of cells.

Ms Uphoff continued her work using toxic radiations to define the conditions governing transplantation of bone marrow to parental strains. During the past year attention was focused on the major role that the "quality" of radiation plays in conditioning the survival of bone marrow cell population. The detection of this unsuspected condition occurred when the machine for generating the radiation was changed and a set of experiments was repeated. The interactions between physical characteristics of irradiation and final biological effects are being investigated and final results are not yet available.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05216-07 LPP

PERIOD COVERED
October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)
Role of Cyclic Nucleotide on In Vivo Tumor Growth Inhibition.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Yoon Sang Cho-Chung	Research Chemist	LPP, NCI
OTHER:	Timothy Clair	Chemist	LPP, NCI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The role of cyclic AMP(cAMP)-binding protein in dibutyryl cyclic AMP(DBcAMP)-induced tumor regression was studied. The results show that cAMP-binding protein that translocates into nuclei during tumor regression is 54,000-dalton protein, the regulatory subunit of protein kinase, type II.

PHS-6040
(Rev. 10-76)

Project Description:

Methods Employed:

i. Tumors: Two cell populations isolated from Walker 256 (W256), hormone-independent mammary carcinoma; one regresses (DBcAMP-responsive) and the other continues to grow (DBcAMP-unresponsive) under DbCAMP treatment, were used.

ii. DBcAMP treatment in vivo: Dibutyryl cAMP was injected 10 mg/0.2 ml 0.85% NaCl/day/200 g rat s.c.

iii. cAMP-binding assay: a) Standard assay: cAMP-binding activity was measured by the competition assay of Gilman at pH 4.5 or 6.5. b) Total binding assay: the competition assay of Gilman was performed at 23° for 3 hr (^{3}H cAMP-binding to proteins includes not only free sites but also endogenously bound sites by the exchange).

iv. Protein kinase assay: the activity was determined by measurement of the incorporation of ^{33}P from γ-labeled ATP into histone.

v. Endogenous phosphorylation: protein kinase dependent endogenous phosphorylation was determined by the incorporation of ^{33}P from γ-labeled ATP into endogenous substrates.

vi. Cell fractionation, column chromatography, and disc gel electrophoresis followed standard techniques.

Major Findings:

I. Identification of cAMP-binding Protein as the Regulatory Subunit of Protein Kinase, Type II, in W256 Mammary Carcinomas.

DEAE-cellulose chromatography (DE52) of cytosols from DBcAMP-responsive W256 rat mammary tumor showed a single major peak of cAMP-dependent protein kinase activity (80% of the total cAMP-dependent protein kinase activity) eluted at a conductivity of 6 mmho, indicating that this tumor possesses mainly type II, protein kinase.

Determination of cAMP binding activity in the column eluate showed a major cAMP-binding activity coincident with the protein kinase peak, demonstrating the relationship between cAMP-binding protein and the regulatory subunit of the kinase.

Measurement of endogenous phosphorylation of the column eluate showed the peak phosphorylation coincided to be protein kinase peak, further indicating that the kinase is type II but not type I.

Following endogenous phosphorylation (autophosphorylation), the regulatory subunit of type II protein kinase from the column eluate was localized by autoradiography on SDS-polyacrylamide gels as 54,000 dalton protein band.

It was observed that the 54,000-dalton regulatory subunit of purified type II protein kinase from bovine heart, co-chromatographed on DEAE-cellulose gels with an endogenously phosphorylated protein of 54,000 daltons from the DBcAMP-responsive W256 tumor cytosol.

In the DBcAMP-unresponsive W256 cytosol, cAMP-dependent type II protein kinase and cAMP-binding activities decreased to 50% of that in the responsive W256 cytosol. The autophosphorylated regulatory subunit of the protein kinase fraction from the unresponsive W256 was also identified as 54,000 dalton protein on SDS-polyacrylamide gels.

II. Nuclear Translocation of Protein Kinase, Type II and W256 Mammary Carcinoma Regression.

6 days following DBcAMP treatment resulted in a marked decrease of cAMP-binding and type II protein kinase activities in the cytoplasm and the concomitant increase of these activities in the nuclei of DBcAMP-responsive W256 as revealed by DEAE-cellulose chromatography, whereas the same treatment produced no change in the binding and kinase activities of the unresonsive W256 cytosol or nuclei.

The increased protein kinase activity in the nuclei of the responsive W256 was no longer stimulated by cAMP but strongly inhibited by protein kinase inhibitor protein, suggesting that the increased kinase activity is due to the catalytic subunit derived from cAMP-dependent protein kinase.

The increased cAMP-binding species in the nuclei of the responsive W256 exhibited no longer endogenous phosphorylation but co-migrated on SDS-polyacrylamide gels with 54,000 dalton regulatory subunit of type II protein kinase from bovine heart, suggesting that the increased cAMP-binding species represent the regulatory subunit of protein kinase, type II.

Incubation of tumor slices from DBcAMP-responsibe W256 with cAMP in vitro resulted in a change in the endogenous phosphorylation of the cytoplasmic and nuclear proteins and the results follows: a) the unincubated DBcAMP-responsive W256 cytosol exhibits two major peaks of phosphoprotein, one with molecular weight of 54,000-dalton and the other with 43,000-dalton as revealed by SDS-polyacrylamide gel electrophoresis: b) following incubation of the tumor slices with 10^{-5}M cAMP at 30° for 10 min, both 54,000 dalton and 43,000-dalton phosphoprotein peaks in the cytosol increased ∿3-fold of that in unincubated tumor cytosol; c) further incubation of the tumor slices resulted in gradual decrease of 54,000-dalton phosphoprotein peak and continous increase of 43,000-dalton phosphoprotein peak in the cytosol; d) in the unincubated tumor nuclei, a low molecular weight (∿12,000 dalton) basic protein species is the major endogenous substrate for nuclear protein kinase, whereas in the nuclei from the incubated tumor slice with cAMP, a new nonhistone basic protein species of a higher molecular weight (∿ 76,000-dalton) became the predominant substrate of the endogenous kinase; e) the phosphorylation of cytoplasmic 54,000-dalton phosphoprotein and nuclear 76,000-dalton phosphoprotein was inversely related during cAMP stimulation of the responsive W256 tumor slices, this relationship, however

was not appreciated following cAMP stimulation of the unresponsive W256 tumor slices.

These results suggest that the lack of nuclear accumulation of protein kinase type II correlates with DBcAMP-unresponsiveness of ono cell population of W256 mammary carcinoma. It is concluded from this study and our previous studies that 54,000-dalton cAMP-binding protein, the regulatory subunit of protein kinase type II may play an indispensable role in cAMP-regulation of tumor growth in vivo.

Significance to Cancer Research and the Program of the Institute: (NCP objective #6, Approach #3).

These studies contribute to the understanding of the molecular mechanism of cAMP-induced tumor regression. Nuclear accumulation of cAMP-binding protein kinase in DBcAMP-responsive W256, following DBcAMP treatment in vivo, incubation of tumor slices with cAMP in vitro, and in the experiments with cell-free systems, strongly suggests the important role of these proteins in cAMP-induced tumor regression. Understanding of molecular mechanism of cAMP action in growth control is essential for the clinical application of cAMP in cancer treatment.

Proposed Course of Research: To extend the investigation on the mechanism of cAMP action on growth control, the following proposal is made: 1) purify protein kinase, type II from DBcAMP-responsive and -unresponsive W256 and characterize the physico-chemical properties of the isolated enzymes and their regulatory and catalytic subunits. 2) Assess nuclear binding of these purified proteins in a cell-free system. 3) Assess nuclear acceptor sites of these macromolecules. 4) Assess the inter-relationships between the regulatory and the catalytic subunits of protein kinase, type II in the nuclei. 5) Assess whether phosphorylation is intimately related with the nuclear binding of these proteins.

Publications:

1. Cho-Chung, Y. S., Clair, T., Yi, P. N., and Parkison, C.: Comparative studies on cyclic AMP binding and protein kinase in cyclic AMP-responsive and -unresponsive Walker 256 mammary carcinomas. J. Biol. Chem. 252: 6335-6341, 1977.

2. Cho-Chung, Y. S., Clair, T., and Porper, R.: Cyclic AMP-binding proteins and protein kinase during regression of Walker 256 mammary carcinoma. J. Biol. Chem. 252, 6335-6341, 1977.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08222-03 LPP

PERIOD COVERED
October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

Interrelationship of Cyclic Nucleotide and Steroid Hormone in Growth Control

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Yoon Sang Cho-Chung	Research Chemist	LPP, NCI
OTHER:	Jeffrey S. Bodwin	Surgeon	LPP, NCI
	Timothy Clair	Chemist	LPP, NCI

COOPERATING UNITS (if any)

None

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SECTION
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NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The intervention of cyclic AMP in the action of estrogen in the growth and regression of hormone-dependent mammary tumors was studied. The results show that tumor regression induced by either ovariectomy or dibutyryl cAMP treatment occurs through a similar biochemical mechanism.

PHS-6040
(Rev. 10-76)

Project Description:

Methods Employed:

i. Tumors: Primary 7,12-dimethylbenz(α)anthracene (DMBA)-induced and N-nitrosomehtylurea (NMU)-induced mammary carcinomas, and transplantable DMBA #1, MT13762 and MTW9A mammary carcinomas were used. Regression was obtained by either bilateral ovariectomy or dibutyryl cyclic AMP (DBcAMP) treatment of host animals (10 mg/0.2 ml, 0.85% NaCl/200 g rat/day s.c.).

ii. Estrogen binding assay: dextran coated charcoal method.

iii. cAMP assay: cyclic AMP lebels in tumors were measured by Gilman's competitive protein binding assay.

iv. cAMP-binding assay: the competition assay of Gilman was performed at 23° for 3 hr.

v. Protein kinase assay: the activity was measured by the determination of the incorporation of ^{33}P from γ-labeled ATP into histone.

vi Adenylate cyclase assay: by the method of Krishna et al ± NaF, utilizing AMP-PNP (ATP analogue) as the substrate.

vii. cAMP-phosphodiesterase assay: used method of Thompson and Appleman.

viii. Cell fractionation, sucrose density gradient centrifugation, and disc gel electrophoresis followed standard techniques.

Major Findings:

I. Estrogen- and cAMP-binding Capacities and Tumor Response to Ovariectomy

Estrogen- and cAMP binding activites were measured in the biopsy specimens of 35 rat mammary tumors and the response of these tumors to host ovariectomy was followed. The results showed that all of the tumors with high estrogen-binding (> 1 pmole/g tissue) and low cAMP-binding (< 80 pmole/g tissue) activities regressed following ovariectomy, whereas tumors with low estrogen-binding and high cAMP-binding activities grew following ovariectomy. Tumors with a high or low binding capacity for both estrogen and cAMP also grew during ovariectomy. Thus the binding capacities of estrogen and cAMP are closely related to the response of tumors to ovariectomy.

II. Estrogen- and cAMP-binding Activities in Regressing DMBA Tumors.

When hormone-dependent DMBA mammary tumors undergo growth arrest following either ovariectomy or DBcAMP treatment of the host, a change in the specific binding of estrogen and cAMP occurs in the tumors. Three days after either ovariectomy or DBcAMP treatment, cAMP-binding increased 5- and 2-fold in the tumor nuclei and cytosols, respectively, whereas nuclear and

cytoplasmic estrogen binding decreased by 70% and 25%, respectively. Cyclic AMP-binding increased initially in the cytosol where it later decreased when the nuclei-associated cAMP-binding reached peak activity. On the contrary, estrogen-binding decreased initially in the nuclei and later in the cytosol. These reciprocal changes in cAMP- and estrogen-binding activities were detectable within 1 day after either ovariectomy or DBcAMP treatment, when there was no appreciable change in tumor size. The changes were reversed, however, when tumor growth was resumed following the injection of estradiol valerate or cessation of DBcAMP treatment.

Concomitant with the increase of cAMP-binding activity in the regressing tumors was the increase in protein kinase activity. The identity between cAMP-dependent protein kinase and cAMP-binding protein as found in DEAE-cellulose column chromatography profiles and sucrose density gradient sedimentation patterns, suggested a similar relationship between these proteins in DMBA tumors as has been shown in other tissues. The increased activities of both cAMP-binding and protein kinase due to ovariectomy could be attributed to new protein synthesis, since cycloheximide was found to block these activities.

Ovariectomy and DBcAMP treatment of DMBA tumor-bearing hosts resulted in an immediate increase in the cAMP content of the respective tumors. A peak level (∿ 3-fold that of the growing tumor) was reached 1 day after DBcAMP treatment and 2 days after ovariectomy. The increase in cAMP content of the tumors followed a sharp increase in adenylate cyclase activity. Three days after either treatment, the cAMP content decreased simultaneously as the cAMP-phosphodiesterase activity increased. The increases in cAMP level, adenylate cyclase and phosphodiesterase activities subsided in those tumors resuming growth following the injection of estradiol valerate or cessation of DBcAMP.

III. Nuclear Protein Phosphorylation in Regressing DMBA Tumors.

A change in protein kinase-dependent phosphate incorporation in isolated nuclei of DMBA tumors was also observed following either ovariectomy or dibutyryl cyclic AMP treatment. A low molecular weight (∿ 12,000 dalton) basic protein species was found to be the major endogenous substrate for nuclei-associated protein kinase in growing tumors, whereas in nuclei of regressing tumors, the radioactivity peaks associated with 12,000-dalton protein species decreased by ∿ 40% and new radioactivity peaks coincident with a higher molecular weight (∿ 76,000-dalton) basic protein species appeared. Phosphorylation of this regression-associated protein (RAP) began within 1 day after either ovariectomy or DBcAMP treatment and stopped when tumor growth was resumed following the injection of estradiol valerate or cessation of DBcAMP treatment.

Whether phosphorylation of RAP is, indeed, related to the effect of cAMP was examined under more clearly defined conditions *in vitro*. When slices from growing DMBA tumors were preincubated with 10^{-5} M cAMP at 30° for 40 min, phosphorylation of RAP was induced, whereas this phosphorylation was

inhibited when 10^{-7} M 17 β-estradiol was added simulataneously with cAMP to the incubation medium. Thus phosphorylation of RAP appears to be related to the specific action of cyclic AMP that counteracts the effect of estrogen in the nuclei of hormone-dependent mammary tumors.

This study presents evidence of an antagonistic action between cAMP and a steroid hormone in the growth control of hormone-dependent mammary tumors - estrogen may promote growth, whereas cAMP may be involved in the regression process.

Significance to Cancer Research and the Program of the Institute: (NCP objective #6, Approach #3)

These studies contribute to the understanding of the mechanism of hormone-dependent growth. The study shows the first evidence that action of estrogen and cAMP may be reciprocally related in the growth control of hormone-dependent mammary tumors, thus would contribute to the better understanding, treatment and prevention of malignant hormone-dependent growth.

Proposed Course of Research:

i. Assess the inter-relationship between cAMP and estrogen in the nuclear-binding in a cell free system.

ii. Assess the nuclear acceptor sites for cAMP- and estrogen binding proteins.

iii. Assess whether a modification of chromatin proteins or DNA would influence the nuclear-binding of cAMP or estrogen.

iv. Assess whether nuclear phosphorylation is inter-related with the nuclear-binding of estrogen or cAMP.

v. Identify regression-associated-protein (RAP).

Publications:

1. Cho-Chung, Y. S., and Redler, B. H.: Dibutyryl cyclic AMP mimics ovariectomy. Growth arrest and phosphorylation of a non-histone nuclear protein in a hormone-dependent mammary tumor. Science 197: 272-275, 1977.

2. Bodwin, J. S., and Cho-Chung, Y. S. Decreased estrogen binding in hormone dependent mammary carcinoma following ovariectomy or dibutyryl cyclic AMP treatment. Cancer Letters 3: 289-294, 1977.

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5. Cho-Chung, Y. S.: Interaction of cyclic AMP and estrogen in tumor growth control. In Sharma, R., and Criss, W.E. (eds): Endocrine Control in Neoplasia. New York, Raven Press, 1978, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08223-02 LPP

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Estrogen Binding Studies in Ovarian Dependent and Independent Rat Mammary Carcinoma.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Jeffrey S. Bodwin	Surgeon	LPP, NCI
OTHER:	Yoon Sang Cho-Chung	Research Chemist	LPP, NCI

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SUMMARY OF WORK (200 words or less - underline keywords)

This study continues to investigate how the specific biochemical event of estrogen receptor binding relates to cAMP binding system and growth and regression of ovarian dependent and independent tumors.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: (1) To characterize and quantitate estrogen binding components in cytosol and nucleus of ovarian dependent and independent tumors. (2) To investigate the biological significance of estrogen binding in relation to growth and regression, due to hormone deprivation or dibutyryl cAMP treatment, in ovarian dependent and independent tumors. (3) To study the relationship of estrogen receptors with other hormone receptors, specifically cAMP binding protein, in the growth and regression of cancer cells. (4) To duplicate *in vitro* using tissue explants and homogenates, biochemical events documented during tumor regression *in vivo*. (5) To study how pharmacological doses of estrogen produce regression of hormone dependent and independent tumors.

Methods:

1) 7,12 DMBA and NMU induced primary rat mammary carcinomas were used as experimental models of hormone dependent tumors.

2) DMBA #1, 13762 and MTW9A transplantable rat mammary carcinomas were used as hormone independent tumors.

3) Estrogen binding was measured by dextran coated charcoal method and whole nuclei exchange technique.

4) *In vivo* radionuclide injection into host animals to determine cytosol and nuclear binding of estrogen.

5) Differential centrifugation and sucrose density gradients were used to isolate and characterize binding of estrogen to subcellular components.

6) Incubation of whole tissue slices with [^{3}H]-17β estradiol to determine cytosol and nuclear incorporation of estrogen.

Major Findings:

1) Estrogen binding in the cytosol of hormone dependent tumors 7,12 DMBA decreases over time during regression due to ovariectomy or dibutyryl cAMP treatment, however, binding components exhibit similar binding affinities (kd) and sedimentative properties in growing or regressing tumors.

2) Binding of estrogen to nuclear components can be quantitated by using dextran coated charcoal assay of KCl extract of nuclei or whole nuclei exchange method. Both methods reveal two sets of nuclear binding sites, high ($kd \sim 5 \times 10^{-10}$) and low ($Kd \sim 5 \times 10^{-5}$) affinity sites.

3) High affinity nuclear binding in 7,12 DMBA tumors decreases to 50% of control value, 6 days after ovariectomy as measured by either DCC assay or whole nuclei exchange method.

4) Pharmocological does of 17β estradiol (100 mg/day produces regression in 7,12 DMBA tumors with a decrease in cytosol estrogen binding and an increase in nuclear binding.

5) Preincubation of whole tissue slices with 17β estradiol results in an increase in nuclear estrogen binding which can be inhibited by simultaneous addition of cAMP with estradiol.

6) Prediction of hormone dependency with subsequent regression after ovariectomy appears to be closely correlated with two biochemical parameters; estrogen receptor and cAMP binding protein.

Significance to Cancer Research: (NCP Objective #6 Approach #3) By investigating the quantitative and qualitative differences in estrogen binding components of hormone dependent and independent tumors, the functional role of the estrogen receptor and its relation to other hormone receptors during growth and regression of tumor cells can be elucidated. Also by duplicating *in vivo* responses *in vitro* and correlating ovarian dependency with biochemical parameters, a clinical application to the treatment of breast cancer can be proposed.

Proposed Course of Research:

1) To correlate estrogen binding capacity of tumor cytosol and nucleus with the other hormonal systems and their relation to ovarian dependency of tumor growth.

2) To investigate the functional role of estradiol nuclear binding during growth and regession and its specific relation to cAMP/protein kinase system and phosphorylation of nuclear proteins.

Publications:

1. Bodwin, J.S. and Cho-Chung, Y.S.: Decreased estrogen binding in hormone-dependent mammary carcinoma following ovariectomy or dibutyryl cyclic AMP treatment. Cancer Letters 3: 289-294, 1978.

2. Bodwin, J.S., Clair, T. and Cho-Chung, Y.S.: Dibutyryl cyclic AMP mimics ovariectomy: Inverse relation between estrogen-receptors and cyclic AMP-binding proteins in hormone-dependent mammary tumor regression. Cancer Research, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08231-02 LPP

PERIOD COVERED
October 1, 1978 through September 30, 1978

TITLE OF PROJECT (80 characters or less)
Synthesis of α-LA and Casein mRNA, during Gestation and Lactation of Rat Mammary Gland

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. L. Nakhasi	Visiting Fellow	LPP, NCI
OTHER:	Pradman K. Qasba	Visiting Scientist	LPP, NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.75	.75	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Measurements of α-LA and casein levels, their messenger activities and mRNA sequences in the gland show that these differentiated functions are expressed with the onset of gestation. Biphasic expression of these proteins during the second and third weeks of gestation is measurable by their protein levels, messenger activities and mRNA sequences. The mRNA levels are, however, out of phase with protein levels during the first week of gestation, but remain in phase thereafter. The results show phasic and sequential expression of these differentiated functions during lactogenesis.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To study the synthesis of α-La and casein mRNA during various stages of gestation and lactation of rat mammary gland and in rat mammary carcinomas.

Methods Employed: The methods being used are detailed in project ZO1 CB 08219-03-LPP.

Major Findings:

I. α-LA and casein protein levels in the rat mammary gland during gestation and lactation.

Using radioimmunoassay for α-LA, 42K, 29K and 25K casein, we have quantitated their levels in mammary gland during various stages of gestation and lactation. These results show that:

1. α-LA and caseins in mammary gland increase during 2nd to 5th day of gestation followed by decrease in their levels till 7th day of gestation. The levels of these differentiated proteins then again increase after 7-9th day of gestation and continue rising till parturition. The protein levels of individual caseins and α-LA in the mammary gland during gestation is 42K>29K>25K>αLA.

2. During lactation α-LA levels in the mammary gland increase in 10 to 20-fold compared to its levels in the 3rd week of gestation; 42K casein increases by about 2-fold, 25K casein by about 12-fold and the levels of 29K casein remain unchanged.

II. α-LA and casein m-RNA activities determined by wheat germ translational systems.

α-LA and casein m-RNA activities in total RNA extracted from the mammary gland at various stages of gestation and lactation were measured by wheat germ translational system and results show that:

1. Total RNA isolated from the mammary gland during the 1st week of gestation showed little mRNA activity. Casein, α-LA and total mRNA activities increase from 7th day of gestation followed by two peaks of activity during the third week of gestation. The first peak of activity corresponds to the 12th day of gestaion and the other to the 17th day of gestation. The ratio of casein to α-LA mRNA actvities during the third week of gestation remains around 1:0.6.

2. The total mRNA activity in the lactating mammary galnd increases until 14th day of lactation and is about ten times higher than gestation. α-LA mRNA activity increases from the 1st day of lactation and plateaus by the 4th day and then declines by the 14th day.

Casein mRNA activity continues to increase until the 8th day where it plateaus and then declines from the 14th day of lactation. Other mRNA activities start appearing from 4th day of lactation, reach a maximum on 14th day and then decline. These results show a sequential synthesis of these messages in the mammary gland.

III. Quantitation of α-LA mRNA in the mammary gland with α-LA cDNA probe during gestation and lactation.

Levels of α-LA mRNA in the total RNA isolated from the rat mammary gland during various stages of gestation and lactation was quantitated by the hybridization of labeled α-LA cDNA probe in the presence of excess RNA. The results show that:

1. α-LA mRNA levels increase during 1st week of gestation reaching a maximum on 5th day and then declines till 9th day of gestation. Messenger RNA levels for α-LA then starts increasing again, reaching a maximum on 17th day of gestation followed by a decline in its levels till parturition.

2. mRNA levels of α-LA increase by about 8-10 fold from the 1st day of lactation until 12th day and then from 14th day the levels decrease going below the levels of 1st day of lactation.

Above results of α-LA protein and mRNA levels in summary show:

a) Within the first week of gestation rat mammary gland undergoes first phase of lactogenic differentiation. Milk proteins-α-lactalbumin (α-LA) and caseins--compared to virgin gland increase by 2-4 fold within 2-5 days of gestation and then decline by about 1/4th to 1/5th. The levels thereafter increase during the second and third week of gestation.

b) α-LA mRNA levels measured with the cDNA probe, synthesized from the mRNA of 4-5 day lactating gland, shows an increase of about 20-fold from the first to the fifth day of gestation. The levels thereafter decline to 1/3 by the seventh day. These mRNA levels are, however, out of phase with the α-LA protein levels, suggesting 1) differences in the turnover of α-LA mRNA and/or α-LA protein; or, 2) differences in the translational efficiency of mRNA during this period.

3. α-LA mRNA per μg of total RNA or per μg of DNA increases after the 9th day of gestation reaching maximum on the 17th day. The message levels compared to the α-LA protein levels, suggest that either this message is less efficiently translated during this period of gestation or -LA protein has a faster turnover.

4. Quantities of α-LA mRNA per g of total RNA does not change from the 19th day of gestation till the 12th day of lactation. They start declining thereafter until the 21st day of lactation. However the total RNA content of the gland increases by about 12-fold from the

day of parturition till 12-14th day of lactation. This suggests that the α-LA mRNA quantites increase proportionately with the rest of the RNA.

5. α-LA mRNA and casein mRNA activities measured by wheat germ translational system show a biphasic activity during the second and third weeks of gestation with peaks of activities corresponding to the 12th and 17th days of gestation. The activities appear sequentially during lactation; α-LA activity plateauing by the fourth to 8th day and the other mRNA activities by the 14th day.

Significance to Cancer Research and the Program of the Institute: Knowledge on the synthesis and processing of α-LA and casein mRNA will help us in understanding the mechanisms involved in the expression of these differentiated functions in normal and neoplastic tissue.

Proposed Course of Research:

1) The turnover of these proteins and mRNA at different stages of development will be measured,

2) using explant cultures the changes of protein and mRNA levels will be correlated with the hormonal changes during these periods of development.

Publications:

1. Qasba, P. K. and Nakhasi, H. L.: Abundance of α-LA mRNA in 4 day lactating rat mammary gland. Purification of rat α-LA mRNA. Proc. Nat. Acad. Sci. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08219-03 LPP

PERIOD COVERED
October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)
Abundance of α-Lactalbumin m-RNA - Isolation and Purification of Rat α-Lactalbumin and Casein mRNA and Synthesis of their cDNA Probes.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Pradman K. Qasba	Visiting Scientist	LPP, NCI
OTHER:	H.L. Nakhasi	Visiting Fellow	LPP, NCI

COOPERATING UNITS (if any)

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Laboratory of Pathophysiology

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NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.50	0.50	

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Rat α-lactalbumin (α-LA) mRNA has been purified from the total RNA of 4-day lactating mammary gland by poly(U)-sepharose chromatography and by fractionation of the poly(A)+ RNA on sucrose gradients. α-LA mRNA was homogeneous as judged by electrophoresis in (a) urea-agarose gel and (b) 1.5% agarose gel under glyoxal-denaturation conditions, which gave a molecular weight of 210,000. The chymotryptic fingerprints of the protein synthesized with this mRNA, in a translational system derived from wheat germ, were similar to those of purified rat α-LA.

In rat milk or lactating gland, the content of 42K casein is 6 times greater than 29K casein and 10 fold greater than α-LA. However, α-LA mRNA (210,000 M.W.) and two casein mRNAs (460,000 M.W. and 390,000 M.W.), coding for 42K and 29K caseins, were present in equal proportions when measured with cDNA probes in the total RNA of 4-5 day lactating gland. Moreover α-LA and total casein were synthesized in a ratio of 1:2.5 in a wheat germ translational system using the total RNA of the lactating gland. The results suggest post-transcription controls in the synthesis of these proteins.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To purify and characterize rat α-lactalbumin and casein mRNA and their complementary DNA probes so that one is able to study the regulation of the synthesis of these messenger RNA's; (a) during development of mammary gland; (b) in neoplastic mammary tissue; and (c) the influence of hormones on their synthesis.

Methods: 1) Isolation of mRNA on Poly-U-sepahrose or dT cellulose columns and further purification on sucrose gradients. 2) In vitro protein synthesizing system derived from wheat germ which is dependent on exogeneous mRNA. The synthesis of specific proteins is measured by immunoprecipitation method.

Major Findings: α-LA and casein mRNA from the lactating mammary gland was quantitated by translating the mRNA in a wheat germ translationsl system and immunoprecipitating proteins with α-LA and casein antiserum. In vitro synthesized α-LA and caseins coelectrophoresed on SDS-polyacrylamide gels with marker α-LA and caseins. Detailed studies showed:

1) α-LA represents 4.5% of and casein 52% of the total protein in rat milk In 3-5 day lactating rat mammary gland, α-LA is 0.6% and caseins 7.5% of the total protein giving a ratio of about 1:0.1 of casein to α-LA proteins.

2) From total cellular RNA, α-LA mRNA activity represents 30% of the total m-RNA activity with casein m-RNA representing the remaining (70%) activity. Thus, the ratio of casein to α-LA m-RNA activity in total RNA is thus 4-5 times more than is expected from the casein to α-LA protein ratios of 3-5 days lactating mammary gland, indicating that either α-LA m-RNA is present in excess quantities or is translated more efficiently in the wheat germ system than the casein m-RNA.

3) In poly A-bearing m-RNA, α-LA represents 40-50% of the total poly A-bearing mRNA activity, and casein represents the remaining 50-60%.

4) After fractionation of poly A-RNA on sucrose gradients, α-LA message sediments as an 8.5 S peak and represents nearly 50% of the total activity. Casein m-RNA activities sediment betwen 12-16 S region of the gradient.

5) After second sucrose gradient centrifugation of active m-RNA fractions, the α-LA and casein m-RNA's have been purified to homogeniety. The analysis of the α-LA mRNA on Agarose-Urea (ph 3.5) gels gave a single band, the mobility of which is slightly slower than α-hemoglobin mRNA. Molecular weight compared with known markers was found to be about $2x10^5$. Individual casein m-RNA's were also separated on sucrose gradients. On agarose-urea gels individual 16 and 12S casein m-RNA's were characterized.

6. We have synthesized the c-DNA probe from α-LA-mRNA using AMV reverse transcriptase. The c-DNA was about 550 nucleotides long measured by sedimentation in alklaine sucrose gradients and on alkaline agarose gels. This represents about 85% of the α-LA mRNA length.

7. The complexity of α-LA m-RNA was compared with the complexity of hemoglobin m-RNA. α-LA c-DNA hybridized with α-LA mRNA at a R_{ot} 1/2 of 2.2×10^{-3} M.s.1^{-1}. Under identical conditions c-DNA probe synthesized from a mixture of α and β-hemoglobin m-RNA hybridized with its m-RNA at a R_{ot} 1/2 of 2×10^{-3}M.s.1^{-1}. This demonstrates that α-LA mRNA has a complexity comparable with the hemoglobin mRNA.

8. The α-LA m-RNA content of 3-5 day lactating mammary gland, measured with α-LA-c-DNA probe, represents 0.5% of the total RNA, which is equal to the amount of 15S casein m-RNA quantitated by Jeffry Rosen at Houston.

Proposed Course of Research: 1) Primary transcripts of α-LA and casein mRNA and their processing will be studied. 2) sequence analysis from 3' and 5' end of the α-LA and casein mRNA's will be studied. 3) Isolate the genes for α-LA and casein.

Significance to Cancer Research and the Program of the Institute: DNA probes for α-LAR casein are very sensitive probes to measure the levels and turnover of mRNA in mammary neoplastic tissue.

Publications:

1. Chakrabartty, P.K. and Qasba, P.K.: Partial purification of rat lactalbumin mRNA. Nucleic Acid Res. 4: 2065-2074, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08218-03 LPP

PERIOD COVERED
October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)
Studies on Hormonal Mechanism(s) Regulating The Expression of Milk Proteins in Normal and Neoplastic Tissue: Purification and Characterization of Rat α-Lactalbumin.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Pradman K. Qasba	Visiting Scientist	LPP, NCI
OTHER:	H.L. Nakhasi	Visiting Fellow	LPP, NCI

COOPERATING UNITS (if any)

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NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.50	0.50	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Two species of α-lactalbumin, α-lactalbumin$_1$ and α-lactalbumin$_2$, were separated from rat milk and purified to homogeneity by gel filtration, followed by DEAE-cellulose chromatography. α-Lactalbumin, shows a molecular weight of 21,500 as determined by sedimentation equilibrium analysis and sodium dodecyl sulfate-polyacrylamide gel analysis. α-Lactalbumin$_2$ has a molecular weight of 16,000 measured by sedimentation analysis. α-Lactalbumin$_2$, however, exhibits abnormally high molecular weight of 22,500 on sodium dodecyl sulfate polyacrylamide gels. Both α-lactalbumins are active in lactose synthase assay and are glycoproteins containing 7 to 9% carbohydrate. Antiserum raised against α-lactalbumin$_1$ cannot discriminate between the two species in a radioimmunoassay.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: Involvement of hormones during the expression of differentiated function has been well documented. Growth of several cell types and tumors has also been shown to be dependent on these hormones. Very little is known, however, about the existence of any relationship, if any, between the differentiated function and growth. Mammary gland, which synthesizes two specific proteins -α -lactalbumin and caseins - at the time of gestation and lactation and the synthesis of which depends on hormones, offers a unique model system to investigate the question of relationship between growth and differentiated function. Several mammary tumors have been shown to be dependent on these hormones for their growth and the removal of the hormones triggers either growth arrest or regression. The present study has been initiated to investigate these questions at the molecular level and for this purpose our objective has been the following:

a) purify and characterize rat α-lactalbumin (Project # Z01 CB 08218-02
b) isolate and purify rat α-lactalbumin and casein messenger RNA. (Project #Z01 CB 08219-02)
c) quantitate the levels of α-lactalbumin and casein and their messenger RNA's during various stages of gestation and lactation of rat mammary gland. (Project #Z01 CB 08217-02)

Methods: α-Lactalbumin was separated from whey proteins on Sephadex G-150 columns and then further purified on DEAE-cellulose column. Purification was monitored by enzymatic methods using cow galactosyl transferase as a protein in a lactose synthetase assay.

A) Properties of Rat α-Lactalbumin

a) Rat α-Lactalbumin

Two species of α-Lactalbumin - αLA_1 and αLA_2 - were separated from rat milk and purified to homogeneity by gel filtration, followed by DEAE-cellulose chromatography. Detailed characterization of the two species of α-LA showed that:

1. Both α-lactalbumins are active in lactose synthase assay and are glycoproteins containing 7-9% carbohydrate.

2. α-LA_1 is a bigger molecule in contrast to other known α-lactalbumins, and has a molecular weight of 21500 as determined by sedimentation equilibrium and SDS-polyacrylamide gel analysis.

3. α-LA_2 has a molecular weight of 16,000 measured by sedimentation analysis, but exhibits abnormally high molecular weight of 22,500 on SDS-polyacrylamide gels.

4. α-LA_1, and α-LA_2 share common c-terminal but differ in their N-terminal amino acids.

5. The peptide maps after chymotryptic digestion show that α-LA_1 contains all the peptides present in α-LA_2 plus five additional peptides.

6. α-LA_1 can be converted to α-LA_2 by autodigestion. (The CNBr cleavage peptides of α-LA_1 and α-LA_2 are currently being analyzed).

Rat α-LA_1 is a unique α-lactalbumin in that it is (a) a glycoprotein (b) a larger molecule than any other known α-lactalbumin and still active in lactose synthase assay and (c) is a precursor of α-LA_2 which is of the size of known α-lactalbumins. We have synthesized rat α-LA in vitro by translating α-LA mRNA isolated from lactating rat mammary gland in wheat germ cell-free system. The in vitro synthesized protein which is not glycosylated coelectrophoreses with α-LA_1 in SDS-polyacrylamide gel and has a molecular weight of 21500.

b) Mouse α-lactalbumin

We are at present looking for a precursor of α-lactalbumin in mouse milk. For this purpose we have purified mouse α-lactalbumin from RIII mouse milk. This α-LA is also bigger molecule than bovine and human α-LA and does not seem to be a glycoprotein.

Proposed Course of Research: 1) Amino acid sequence of the two α-LA will be carried out in collaboration with Dr. K. Brew, Department of Biochemistry, Medical School, University of Florida, Miami.

Significance to Cancer Research and the Program of the Institute: Characterization of α-lactalbumin will help us in understanding the mechanism involved in the synthesis of this differentiated function in normal and neoplastic tissue.

Publication:

1. Qasba, P.K. and Chakrabartty, P.K.: Purification and properties of two forms of rat α-lactalbumin. J. Biol Chem. 253: 1167-1173, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08217-04 LPP

PERIOD COVERED
October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)
Synthesis of Mammary Specific Proteins - α-Lactalbumin and Casein in Mammary Carcinomas.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Pradman K. Qasba	Visiting Scientist	LPP, NCI
OTHER:	H. L. Nakhasi	Visiting Fellow	LPP, NCI
	P. M. Gullino	Chief	LPP, NCI
	I. Losonczy	Chemist	LPP, NCI

COOPERATING UNITS (if any)
None

LAB/BRANCH
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NCI, NIH, Bethesda, Maryland, 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.75	0.50	0.25

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Primary carcinomas induced in rats by 7-12 dimethylbenzanthracene (DMBA) or methylnitrosourea (MNU) contain α-LA in quantities equal to the amounts found in the rat mammary gland during 1st to 2nd day of gestation. Among transplantable tumors tested, only R3230 AC and MTW9 but not in DMBA #1, Ascites 13762 and MTW9A transplantable mammary carcinomas. Transplantation of a pituitary gland under the kidney capsule of the host (a) increased αLA content of primary DMBA induced mammary carcinomas, (b) reduced αLA content of primary MNU induced mammary carcinomas and (c) was unable to modify the αLA levels in 5 transplantable mammary carcinomas.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To study the production of α-lactalbumin and casein in mammary carcinoma cells and the influence of hormones on their synthesis in the neoplastic tissue.

Methods:

1) The quantity of casein or αLA in the unknown sample was determined by competitive inhibition assay, i.e. casein or αLA in the sample competed with the binding of the ^{125}I-labeled casein or αLA, thus quantification was based on the reduction of precipitated counts.

2) α-LA and casein mRNA are quantitated by hybridization with their cDNA probes.

Major Findings:

1. Primary mammary carinomas induced by 7-12-DMBA or MNU contain α-lactalbumin, 42K, 29K and 25K caseins. The quantities of these proteins in these tumors are within the levels found in the rat mammary gland during the first and second day of gestation. Among transplantable tumors tested, only MTW9 and R3230AC tumors showed the presence of these proteins and the quantities are nearly two times higher than the amounts found in MNU and 7-12-DMBA tumors. In MNU and 7-12-DMBA induced tumors the levels of individual caseins and α-LA proteins is 42K>α-LA>29K>25K and in MTW9 and R3230AC tumors is 42K>29K>αLA>25K. During the gestation the levels of casein and α-LA in the mammary gland is 42K>29K>25K>α-LA. The results suggest that the synthesis of individual caseins and α-LA's in these tumors is altered, compared to mammary gland during gestation and lactation.

The transplantation of a pituitary gland under the kidney capsule of the host (i) increased α-LA content of primary 7-12 DMBA induced mammary carcinomas (ii), reduced α-LA content of primary MNU tumors, and (iii) did not modify the levels of five transplantable tumors.

2. α-LA mRNA levels in rat mammary tumors were measured with the cDNA probe. In MTW9 and R3230AC tumors the levels are comparable with the mammary gland RNA from 2nd or 3rd day of gestation. α-LA mRNA levels in 7-12 DMBA or MNU tumors are 10-50 fold lower than in MTW9 or R3230AC tumors. Higher levels of αLA protein and low steady state levels of α-LA mRNA suggests faster turnover of the message in these tumors compared to MTW9 and R3230AC tumors.

Influence on the steady state levels of α-LA mRNA in 7-12 DMBA and MNU tumors after implantation of the pituitary under the kidney capsule and during regression of MTW9 tumors are being investigated.

Significance to Cancer Research and the Program of the Institute: Understanding the response of several mammary tumors to various hormonal manipulations will help in designing experiments for early detection and appropriate treatment of breast cancer.

Proposed Course of Research: Turnover of these proteins and mRNA during growth and regression of these tumors will be studied.

Publications:

1. Qasba, P.K. and Gullino, P.M.: α-Lactalbumin content of rat mammary carcinomas and the effect of pituitary stimulation. Cancer Research 37: 3792-3795, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 CB 08226-02 LPP

PERIOD COVERED

October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

The Role of Thyroid Hormones in Growth and Differentiation of Mouse Mammary Glands

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B. K. Vonderhaar	Expert	LPP, NCI
OTHERS:	A. Bhattacharya	Visiting Fellow	LPP, NCI
	A. E. Greco	Chemist	LPP, NCI
	P. L. Russell	Biologist	LPP, NCI

COOPERATING UNITS (if any)

Laboratory of Molecular Biology, DCBD, NCI
Laboratory of Basal Metabolism, NIAMDD
Department of Cell Biology, Baylor College of Medicine

LAB/BRANCH

Laboratory of Pathophysiology

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3.5	2.0	1.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to understand the mechanisms by which milk-protein production in mouse mammary tissue is controlled by various hormones with particular emphasis on prolactin-thyroid hormone interactions. Studies include: 1) evaluation of the cytological and ultrastructural development of virgin mouse mammary tissue after explant culture in the presence of insulin, hydrocortisone, prolactin and thyroid hormones; 2) examination of biochemical and ultrastructural development of these tissues in the presence and absence of DNA synthesis in vitro; 3) characterization of thyroid hormone and prolactin receptors within mammary tissue and their interrelationship; 4) examination of the role that prostaglandins may play in milk-protein production with emphasis on regulation by thyroid hormones and prolactin.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: The purpose of these studies was to define the biochemical mechanism by which milk-protein production in virgin mouse mammary tissue is coupled to traversal of the cell cycle in vitro and the role that thyroid hormones play in this process. The cells from virgin animals cultured in the presence and absence of thyroid hormones were to be examined for cytological, ultrastructural and biochemical development in the presence and absence of DNA synthesis inhibitors. Furthermore, we wished to complete the characterization of the thyroid hormone receptors in mouse mammary glands and investigate the role they play in controlling prolactin binding to this tissue. The role of prostaglandins in the mechanism by which thyroid hormone-prolactin interactions control milk-protein production was to be investigated.

Methods Employed: C3H/HeN mice in various stages of development were used. Animals were made mildly hypothyroid by adding 0.1% thiouracil to the drinking water. Hyperthyroidism was attained by adding thyroxine ($L\text{-}T_4$) to the drinking water at 2 µg/ml. The drug and hormone treatments were maintained for various periods of time depending on the design of the experiment. Two types of organ culture systems were used: the standard explant system and a recently developed strip culture method (Harbell, J. W. et al., In Vitro 12: 490 (1977)). In both cases, tissue was cultured in chemically defined serum-free medium supplemented with various hormones, prostaglandins and metabolic inhibitors. Milk-protein synthesis, mRNA accumulation, cytological and ultrastructural development and prolactin receptor induction were studied. α-Lactalbumin was assayed for enzymatic activity by the production of ^{14}C-lactose from glucose and UDP-^{14}C-galactose. Casein phosphoproteins were assayed by precipitation with rennin-Ca^{++}. Casein peptides were assayed by indirect immunoprecipitation with casein specific antibody and casein mRNA measured by cDNA excess hybridization in the laboratory of Dr. Jeffrey M. Rosen, Baylor College of Medicine. Cytological and ultrastructural development was assessed by light and transmission electron microscopy by Dr. Gilbert H. Smith, LMB, DCBD, NCI. Receptors for thyroid hormones and prolactin were studied by standard techniques.

Major Findings: Mammary tissue from adult virgin euthyroid animals, when placed in organ culture in the presence of insulin (I), hydrocortisone, (F) and prolactin (PRL) produce the milk-proteins casein and α-lactalbumin. This process is dependent on a prior round of cell division in vitro. The lack of detectable α-lactalbumin activity or rennin-Ca^{++} precipitable casein phosphoproteins in mature virgin tissue is not due to cryptic forms of the milk-proteins as extraction with Triton X-100 did not result in their detection. Tissue from glands actively proliferating in vivo (adolescent and pregnant) or biochemically dormant primiparous glands do not need DNA synthesis in order to produce milk. Other "proliferative" mammary cells were examined for their dependence on DNA synthesis to terminally differentiate. Virgin mammary tissue induced to proliferate by in vivo exposure to high levels of thyroxine (hyperthyroid) did not need DNA synthesis in vitro in order to make milk. This is true even when the excess hormone was removed for periods up to 5 weeks and the glands had reverted to a biochemically dormant state before culture. Proliferation was also induced in vivo by injecting virgin animals with prolactin or human placental lactogen for a period of 2 weeks. Withdrawal of

the hormones for 7 more weeks, resulted in a biochemically dormant gland which made α-lactalbumin and casein in vitro in the presence of I, F, and PRL in the absence of DNA synthesis. If these hormone-injected animals were placed on thiouracil to make them hypothyroid during the period of hormone withdrawal they revert to a state of dependence on DNA synthesis just as seen with glands from hypothyroid primiparous animals. Treatment with thiouracil during the final week of involution only, was not sufficient to convert primiparous tissue back to dependence on DNA synthesis.

More detailed examination of the role that DNA synthesis plays in terminal differentiation of virgin mouse mammary tissue centered on examination of cell pellets from collagenase treated tissue using either light or electron microscopy. In all studies parallel biochemical analyses were performed to determine that DNA synthesis as well as α-lactalbumin activity and casein phosphoprotein synthesis were blocked. These studies showed that epithelial cells from uncultured virgin tissue and from tissue cultured in the presence of I alone were primitive in appearance with a small cytoplasmic to nuclear ration (C/N) and no evidence of secretory drops or lipids. Cells cultured in I and F showed the presence of some lipid droplets, rough endoplasmic reticulum (RER) and a larger C/N. Culture in the presence of I, F and PRL, resulted in cells with a large C/N, a rich supply of distended RER, electron dense material in secretory granules and Golgi vacuoles and material in the lumen. Addition of triiodo-L-thyronine (L-T_3) to cultures in the presence of I, F, and PRL resulted in more secretory granules per cell and material in the lumen. Cells cultured in the presence of the various hormone combinations but blocked from traversal of the cell cycle by addition of cytosine arabinoside (araC) could not be distinguished from their unblocked counterparts.

From these observations we concluded that morphological and cytological development can occur in the presence of I, F and PRL without DNA synthesis even though the cells do not make enzymatically active α-lactalbumin or rennin Ca^{++} precipitable casein phosphoproteins.

These data suggested that the biochemical assays employed may require complete processing of milk-proteins (i.e.: casein phosphorylation or conversion of α-lactalbumin from an enzymatically inactive to active form) thus accounting for the inability to detect milk-proteins despite the secretory appearance of the cells.

Explants from virgin tissue cultured in various hormonal systems ± araC were labeled with ^3H-proline and assayed for casein phosphoproteins, casein mRNA and casein peptides. In systems synthesizing no detectable rennin-Ca^{++} precipitable casein, a four-fold increase in casein mRNA was detected in both IFPRL cells and IFPRL+araC cells when compared to IF cells. Addition of T_3 to either of these cultures did not appreciably increase the levels of casein mRNA.

Analysis of casein peptides by indirect immunoprecipitation with casein antibody showed that in the systems prevented from undergoing DNA synthesis in vitro, the mRNA is translated, but at about 1/4 the efficiency of the

unblocked system. Addition of T_3 to the IFPRL system resulted in approximately a 30% increase in the level of casein peptides present.

These data suggest that at least in terms of casein synthesis the block in differentiation due to lack of DNA synthesis in virgin tissue *in vivo* is post-transcriptional and possibly post-translational as well. Experiments in which addition of T_3 was delayed until after 72 hr of culture in the presence of IFPRL and araC suggest that for α-lactalbumin activity, the control by thyroid hormones may also be post-translational.

The molecular mechanism by which thyroid hormones exert their influence on terminal differentiation of mouse mammary glands through interaction with cellular receptors was further investigated. Specific binding proteins for L-T_3 were found in mammary glands from euthyroid virgin and mid-pregnant mice. Binding proteins were found primarily in nuclei and cytosol. Studies have centered on characterization of the cytosol component. Under proper incubation conditions, cytosol receptors are protease digestible and heat labile. They are excluded from a Sephacryl S200 column and are not dissociable into subunits by treatment with either KCN or high salt. The cytosol binding protein is distinct from serum binding protein (TBG) by several criteria including hormone specificity (T_3 preferred over T_4), behavior on gel chromatography, affinity constants, and sensitivity towards trypsin. The cytoplasmic binding protein is activated by trypsin while TBG is inactivated. From Scatchard analysis of binding data it is clear that trypsin increases the number of cytosol binding sites without changing the affinity constant.

Since mammary glands consist of epithelial cells embedded in a fat pad, we investigated the presence of receptors in fat cell cytosol. Cytosol prepared from epithelial-free mammary fat pads of euthyroid virgin glands show a specific binding protein with an affinity for L-T_3 ($Ka \sim 10^6\ M^{-1}$). The parametrial fat pad (white fat which also supports growth of transplanted mammary cells) shows a similar affinity constant. This binding constant differs from that found in intact (epithelial and fat cells) mammary tissue ($Ka \sim 10^7\ M^{-1}$). The high value for the number of binding sites in fat cell cytosol (>100 pmoles/mg protein) coupled with its lower affinity for L-T_3 suggests that the fat cells may act as a storage site for thyroid hormones in the mammary gland.

Previously we had demonstrated that the ratio of thyroid hormones to prolactin is important in the induction of milk-protein synthesis. Thyroid hormones appear to act by modulating the cellular sensitivity towards prolactin. Using an *in vitro* prolactin binding assay to crude membrane fractions or uptake by explants, we observed that glands from mature hypothyroid virgins have little or no detectable PRL binding. On the other hand, glands from hyperthyroid virgins show approximately twice as much binding per mg protein as euthyroid animals. Similar results were obtained with primiparous animals in various thyroid states. Since these primiparous tissues are morphologically indistinguishable, the possibility that variable PRL binding in different thyroid states is related to the differences in the ratio of ducts to alveoli is ruled out. Using epithelial-free fat pads from euthyroid virgin animals, no PRL binding was observed.

We have made attempts to study changes in PRL binding induced by T_3 in vitro using the organ culture techniques. Preliminary observations show that T_3 may induce the PRL receptors in vitro. This induction is blocked by cycloheximide. When T_3 is added directly to crude membranes prepared from lactating mammary glands there was a 50% increase in PRL binding. This increase was not observed with an equal concentration of T_4. The increase was only observed with mammary gland membranes and not with liver membranes.

Prostaglandins have been implicated in prolactin action in several systems. The role of prostaglandins in milk-protein production was examined. The induction of α-lactalbumin activity was enhanced up to 3-fold by the addition of prostaglandin F2α(PgF2α) to cultures of euthyroid virgin or pregnant mammary tissue in the presence of I, F and PRL. Maximum effects were obtained at 10 μg/ml). Prostaglandin E_1 (50 μg/ml) was also active, but PgE_2 and arachadonic acid were not. PgF2α does not substitute for prolactin. It appears to enhance the action of prolactin. The effect of PgF2α was primarily on α-lactalbumin activity. Little increase in casein synthesis was observed. The effect of PgF2α were similar to those seen with addition of thyroid hormones to cultures containing I,F and PRL. Like thyroid hormones, the presence of PgF2α overcomes the need for DNA synthesis in vitro for mature virgin glands to demonstrate induction of milk-proteins. PgF2α also mimics T_3 in overcoming progesterone inhibition of α-lactalbumin synthesis in pregnant mouse mammary tissue in vitro. The effects of PgF2α and T_3 in induction of α-lactalbumin in mid-pregnant tissue are not additive. Initial experiments with indomethacin suggest a partial block of the T_3 effect by this inhibitor of prostaglandin synthesis. These data suggest that T_3 may act on α-lactalbumin synthesis by enhancing prostaglandin synthesis and/or release.

Significance to Cancer Research and the Program of the Institute:
The bulk of evidence in the literature suggests an increased risk of human breast cancer associated with hypothyroidism. However, this risk factor is not clearly established since conflicting observations have also been found. Similarly conflicting results have been reported for both the rat and mouse model systems. For this reason, all aspects of thyroid hormone control of growth and differentiation of mammary tissue must be understood in several model systems. Another risk factor in human breast disease is age at first birth. Early age at first pregnancy decreases risk of subsequent mammary cancer. By examining the hormonal control of development of both virgin and primiparous animals we hope to gain insights into the mechanisms by which this risk factor is controlled.

Proposed Course of Research: The fundamental differences between adult virgin and primiparous animals will be examined. Special emphasis will be placed on control of milk-protein production in cells prevented from traversing the cell cycle in vitro. Collaborative efforts with Dr. Gilbert Smith will continue using an immunoperoxidase method combined with electron microscopy to localize those cells containing casein and α-lactalbumin and correlate this with cells actively making DNA. We are currently relying on others to supply the casein and α-lactablumin antibodies for these studies. However, during the next year we will develop these reagents ourselves. We hope to

expand these studies to the rat model system. The role of thyroid hormones in this system will be investigated with special emphasis on control of prolactin receptors. Differences in plasma membrane composition between liver and mammary glands from animals in various thyroid states will be examined with respect to control of prolactin binding. Studies on the role of thyroid status in mammary tumor induction in both rats and mice will be completed. Induction of prostaglandin synthesis and release in the presence of thyroid hormones will be explored.

Pulbications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08237 01 LPP

PERIOD COVERED
October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

Role of Retinyl Acetate in Preventing Mammary Carcinomas in C3H-AVY Mice

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Antonio Maiorana	Visiting Fellow	LPP, NCI
OTHER:	Pietro M. Gullino	Chief	LPP, NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Experimental work has shown that retinyl acetate (a synthetic derivative of vitamin A) is active in preventing the development of mammary carcinomas, induced in rat after DMBA or NMU treatment. This work aims at establishing whether the same compound can reduce the frequency of mammary carcinomas in C3H-AVY mice, a strain highly susceptible to spontaneous mammary tumors.

PHS-6040
(Rev. 10-76)

Project Description:

Methods: Female mice of C3H-A^{vy} strain are fed retinyl acetate blended in the stock diet at 3 different concentrations (83,41,21 mg per Kg diet). Animals are placed on the experimental diet either since conception (feeding the mother a diet already containing retinyl acetate) or at weaning age or at 3 months of age. All mice are weighed and palpated twice a week for detection of tumors. Complete autopsy is performed at one month after tumor appearance and toxic effects are evaluated.

Major Findings:

1) Control animals showed a mammary tumor incidence of 82%.

2) Almost 75% of mice fed retinoids since weaning age or since 3 months of age, have developed mammary carcinomas.

3) The frequency of mammary tumors in mice fed retinoids from the time of conception has not yet been established.

Significance to Cancer Research and the Project of the Institute: This work will establish whether retinyl acetate can be useful in preventing the development of spontaneous mammary carcinomas in C3H-A^{vy} mice. Demonstration that this compound is active, might improve possibilities for prevention of human breast cancer.

Proposed Course of Research: To complete the project as outlined. Continuation using other vitamin A derivatives will be evaluated on the basis of work in rats now ongoing in Dr. M. Sporn's laboratory.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08227-02 LPP

PERIOD COVERED

October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

Heat Transfer in Neoplastic Tissues

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P.M. Gullino	Chief	LPP, NCI
OTHER:	F. Grantham	Biological Laboratory Technician	LPP, NCI

COOPERATING UNITS (if any)

Dr. Rakesh Kumar Jain, Department of Chemical Engineering
Columbia University, New York, N.Y.

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.20	0.20	2.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objective was to study the events that regulate or interfere with the thermal conductivity of neoplastic tissues and to assess the differences in local perfusion of tumors. The thermal conductivity of Walker carcinoma was found to be 2.8±0.8 MW/cm/°C under physiological conditions. The thermal conductivity varied proportionally to the square root of the perfusion rate when the blood supply to the tumor was doubled or reduced to one-half. The tumor preparation necessary to simultaneously measure temperatures in different sections of the tumor has been developed and the procedure to evaluate changes due to sudden variations in blood temperature has been completed. Determinations aimed at evaluating the loco-regional irroration of the neoplastic tissue are ongoing.

PHS-6040
(Rev. 10-76)

Project Description:

Objective: To calculate through direct measurement the thermal conductivity of a neoplastic tissue (Walker carcinoma) and to assess the role that changes in blood supply have on the thermal conductivity.

Methods: An experimental procedure was developed whereby a thermistor probe and a thermal source were incorporated by a growing tumor. Then an appropriate amount of heat was generated on the surface of the thermal source and the variations in temperature were registered by the thermistor, all incorporated within the tissue and at a known distance for the heat source. Measurements were conducted when blood supply was either in a physiological range or sharply increased or decreased.

Another set of conditions was developed whereby 3 thermistors were incorporated by the same tumor connected to the host by a single artery. A microcatheter was developed to cannulate the tumor artery and to inject a pulse of cold plasma into the tumor in vivo. This procedure permits (a) registration of changes in temperature at 3 different sites of the same tumor, and (b) assessment of the differences in the efficiency of tissue irroration within the same tumor.

Major Findings: (1) The thermal conductivity of Walker 256 mammary carcinoma is 2.8 MW/cm/°C (2) the thermal conductivity varied proportionally to the square root of the perfusion rate when the blood flow was doubled or reduced to one-half the physiological level.

Significance for Biomedical Research and The Program of The Institute: Present day treatment of breast cancer is most effective when applied at very early stages of tumor development. Thermography could be the most appropriate tool for early diagnosis if more basic physiological knowledge about heat source and transfer in normal and neoplastic tissues were available. This work aims at providing this basic knowledge.

Proposed Course of Research: (1) To complete measurements on heat transfer, (2) to complete evaluation of loco-regional differences in tissue irroration, (3) to establish by direct measurement the role of blood supply and metabolic conditions in the heat generation and heat transfer in tumors.

Publications:

Gullino, P.M., Yi, P-N., and Grantham: Relationship between temperature and blood supply or consumption of oxygen and glucose by rat mammary carcinomas. J. Natl. Cancer Inst. 60: 835-847, 1978.

Jain, R.K. and Gullino, P.M.: Heat transfer in neoplastic tissues. In: Advances in Bioengineering, E.S. Grood and C.R. Smith (Eds), New York, American Society of Mechanical Engineers, 1977, pp. 99-100.

Jain, R.K. and Gullino, P.M.: Analysis of transient temperature distributions in a perfused medium due to a spherical heat source with application to heat transfer in tumors: Homogeneous and infinite medium. Am. Inst. Chem. Eng. J., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08215-04 LPP

PERIOD COVERED

October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

Detection of Angiogenic Activity in the Interstitial Fluid of Tumors and Purification of the Angiogenesis Factor(s).

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Antonio Maiorana	Visiting Fellow	LPP, NCI
OTHER:	Pietro M. Gullino	Chief	LPP, NCI

COOPERATING UNITS (if any)

Dr. D.T. Liu - Biochemistry Branch, Division of Bacterial Products
Bureau of Biologics

LAB/BRANCH

Laboratory of Pathophysiology

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Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.9	0.9	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Previous studies have indicated that acquisition of angiogenic capacity may be utilized to predict high risk of neoplastic transformation. Present attempts aimed at: (1) detecting presence of angiogenesis factor(s) in the tumor interstitial fluid (2) purifying and developing a RIA for the factor(s) as a tool for predicting high risk of neoplastic transformation in tissues morphologically not suspected.

Project Description:

Objectives: 1) to determine whether the interstitial fluid of tumors in vivo has any angiogenic activity 2) to extract the antiogenesis factors from the tumor interstitial fluid 3) to obtain an antibody against the purified tumor angiogenesis factor for the development of a RIA.

Methods: The interstitial fluid of tumors is sampled with a technique previously developed. The fluid is fractionated and the lyophilized fractions are included in an inert polymer and implanted onto the rabbit cornea. Development of neovascularization is monitored through daily observation with the slit lamp stereomicroscope.

Major findings:

1) The interstitial fluid of tumors in vivo has a very strong angiogenic activity.

2) The possibility of extracting the angiogenesis factor(s) and developing an antibody against it is still uncertain.

Significance to Cancer Research and the Program of the Institute: The development of a RIA. for the angiogenesis factor(s) should provide an efficient tool for establishing whether a hyperplastic tissue is at higher risk of becoming neoplastic. The application of this procedure to the large number of mammary biopsies should permit detection of high risk populations and contribute to prevention of mammary carcinomas.

Proposed Course of Research: To complete the study as outlined in the objectives.

Publications:

Gullino, P.M.: Considerations on the preneoplastic lesions of the mammary gland. Am. J. Pathol. 89: 413-430, 1977.

Brem, S.S., Jensen, H.M., and Gullino, P.M.: Angiogenesis as a marker of neoplastic and preneoplastic lesions of the human breast. Cancer 41: 239-244, 1978.

Gullino, P.M.: Angiogenesis and oncogenesis. J. Natl Cancer Inst., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05211-07 LPP

PERIOD COVERED
October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

Modification of Histone H1 by Poly(ADPR) in HeLa Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.	P.R. Stone	Visiting Fellow	LPP, NCI
Other:	W.R. Kidwell	Head, Cell Cycle Regulation Section	LPP, NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Cell Cycle Regulation Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014.

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Previous experiments have revealed that nuclei of eukaryotic cells contain an enzyme which polymerises the ADP-ribose moiety of NAD through a 1'-2'-ribose-ribose linkage to form poly(ADP-ribose). The polymer is at least in part associated with basic chromosomal proteins and in particular histone H1. The current objective is to characterize the nuclear proteins to which poly(ADP-ribose) is attached (with special emphasis on histone H1) and to elucidate the significance of this protein - poly(ADP-ribose) association in so far as nuclear processes during cellular growth is concerned.

Project Description:

Methods Employed: Nuclei or chromatin is isolated from HeLa cells and incubated with ^{3}H-NAD, under varying conditions to produce poly ADP-ribosylated nuclear proteins. Selective extraction of the H1-associated poly (ADP-ribose) was achieved by virtue of the differential solubility of H1 in 5% perchloric acid and its insolubility in 18% TCA. Subsequent analysis of the H1 preparation was performed using SDS polyacrylamide and acid-urea polyacrylamide gel electrophoresis as well as ion-exchange chromatography.

Major Findings: Chromatin bound poly (ADP-ribose polymerase attaches polymer chains to histone H1 producing a complex composed of two H1 molecules cross-linked by a single polymer chain. Preparations of H1 histone from HeLa cell nuclei or chromatin incubated with ^{3}H-NAD to permit poly (ADP-ribose) synthesis were electrophoresed on polyacrylamide gels. The incorporated radioactivity migrated as a sharply defined peak in association with a protein band which moved more slowly than H1, the major protein component. On the basis of the identification of the protein component of this complex as histone H1, a value of 15 for the average chain length of the polymer in the complex and a ratio of 7-8 ADP-ribose residues/one H1 molecule in the complex, we have previously concluded that the complex consists of two H1 molecules cross-linked by a single chain of polymer of length 15 residues.

The H1 and poly (ADP-ribose) are specifically packaged within the complex. The rate of poly (ADP-ribose) glycohydrolase-catalysed hydrolysis of the polymer in the H1-complex is only 1/9 that of free polymer, indicating that the polymer is in a protected environment within the complex. The hydrolysis of free polymer in the presence or absence of H1 is the same indiciating a specific mode of packaging of the polymer and H1 within the complex. This is further indicated by the fact that alkaline dissociation of the complex followed by neutralisation markedly exposes the associated polymer to the glycohydrolase.

The H1-polymer complex binds to DNA and exposes the polymer. The complex partially unfolds when it binds to DNA as evidenced by a 2-fold increase in the rate of hydrolysis of the polymer by poly(ADP-ribose) glycohydrolase. Also the H1-polymer complex has been shown to bind to DNA-cellulose (both to single- and double-stranded) as well as does unmodified H1 suggesting a packaging of the polymer in such a way as to not affect the binding of the H1 to DNA. This effect of DNA in stimulating degradation of the H1-polymer complex by glycohydrolase is due to binding of the complex to DNA and not due to an effect of DNA upon the glycohydrolase since free DNA is actually inhibitory to the enzyme, especially single-stranded DNA. This inhibition of the glycohydrolase results from the binding of the DNA to the enzyme and conditions which decrease this binding (e.g. increased ionic strength or addition of H1 which competes for DNA binding) release this DNA inhibition.

Conformational changes of chromatin selectively promote H1- poly(ADP-ribose) complex formation. When the polyamines spermine, spermidine or putrescine at 1 or 2mM are added to HeLa cell nuclei, there is a 1-3 fold differential stimulation of ^{3}H-NAD incorporation into the H1- polymer complex with little effect on total polymer synthesis. At higher polyamine levels, total polymer synthesis is inhibited, but the fraction of polymer associated with H1 is still differentially increased. A differential stimulation of complex synthesis is also produced by the addition of elevated levels of Mg^{++} or Ca^{++}. These results are not due to an effect of these agents on recovery or turnover of the H1-polymer complex but are related to the chromatin condensing action of these agents since enhanced formation of the H1-polymer complex and of chromatin aggregation are closely correlated.

Significance to Biomedical Research and the Program of the Institute: (NCP Objective #6, Approach #3). Understanding the mechanisms whereby nuclear processes, such as chromatin condensation are controlled during cell growth are vital to understanding cell cycle controls and rate limiting steps in cells which are lost upon transformation. The results presented suggest that poly(ADP-ribose) serves a role in modifying the structure of the chromosomal fiber by acting as a bridge between proximal folds of the fiber thus acting as a local, transient fixative. Such a process appears to be essential to progression of the cell to division since inhibitors of polymer synthesis produce cell death.

Proposed Course: It is clear that the poly(ADP-ribose)-H1 complex must be demonstrated to exist *in vivo*. To this end we have begun to directly isolate H1-histone from ^{3}H-adenosine labeled cells using 5% perchloric acid. It is clear that there is very little material and since it is known that the polymer turns over rapidly *in vivo*, from measurements made on the mass of polymer in synchronously growing HeLa cells, one needs to manipulate this glycohydrolase mediated *in vivo* polymer degradation. Thus one direction will be to measure glycohydrolase activity in HeLa cells during the cell cycle and to attempt to find a specific inhibitor of this enzyme in order to quantitate the amount of the H1-polymer complex in HeLa cells during the cell cycle. Such data are vital in elucidating the functional role of the H1-polymer complex in the nucleus and its significance in cellular growth.

Publications:

1. Lorimer, W.S.III., Stone, P.R., and Kidwell, W.R.: Control of Histone H1 Dimer-Poly(ADP-Ribose) Complex Formation by Poly(ADP-Ribose) Glycohydrolase. Exp. Cell Res. 106: 261-266, 1977.

2. Stone, P.R., Lorimer, W.S. III., and Kidwell, W.R.: Properties of the Complex between Histone H1 and Poly(ADP-Ribose) synthesised in HeLa cell nuclei. Eur. J. Biochem. 81: 9-18, 1977.

3. Stone, P.R., Lorimer, W.S.III., Ranchalis, J., Danley, M., and Kidwell, W.R.: Effect of DNA on Poly(ADP-Ribose) Glycohydrolase and the degradation of Histone H1-Poly(ADP-Ribose) Complex from HeLa cell nuclei. Nucleic Acids Res. 5: 173-184, 1978.

4. Byrne, R.H., Stone, P.R., and Kidwell, W.R.: Effect of Polyamines and divalent cations on Histone H1-Poly(ADP-Ribose) Complex Formation. Exp. Cell Res--Accepted for publication.

5. Hilz, H., Adamietz, P., Stone, P.R., Bredehorst, R., and Philips, C.: ADP-ribosylation of nuclear proteins. In: Kent, P.W. (Ed): New Approaches to Genetics. Oriel Press, London, 1978, pp. 126-139.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08229-02 LPP

PERIOD COVERED
October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

Role of Dietary Lipids in Increased Incidence of Mammary Cancer

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	William R. Kidwell	Head, Cell Cycle Regulation Section	LPP, NCI
	Max Wicha	Research Associate	LPP, NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Cell Cycle Regulation Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Epidemiological and experimental studies have implicated high dietary fat in the increased incidence of mammary cancer. The fact that changes in dietary intake of lipids is reflected by comparable changes in the lipid matrix of the mammary gland suggests that fatty acids may have a direct role in mammary carcinogenesis. Experimental systems have been established for assessing fatty acid effects on the growth of cells from normal tissue and carcinogen induced mammary tumors of rats. The studies show that all unsaturated fatty acids present in the gland in appreciable amounts promote cell growth. Each fatty acid has its own characteristic concentration optima, which with normal mammary cells, correlates with the abundance of that fatty acid in the gland. Both normal and tumor cells are inhibited by saturated fatty acids. Maximum growth rate of tumor cells is obtained with oleic acid while normal cells grow best in the presence of linoleic or linolenic acid.

PHS-6040
(Rev. 10-76)

Project Description:

Methods: Ductal fragments and alveolar tissue were isolated from perphenazine treated virgin rat mammary glands by limited collagenase treatment followed by Ficoll density gradient fractionation. Tumor cell aggregates from 7,12-dimethylbenzanthracine induced tumors were similarly prepared. Cells were cultured in standard tissue cultured medium supplemented with whole or lipid-stripped serum plus insulin, prolactin, hydrocortisone, estradiol and progesterone. Cell growth effects of hormones and lipids were assessed by counting cells or by analysis of ^{3}H-Tdr incorporation. Collagen (type IV), basal lamina glycoproteins and α-lactalbumin were quantitated by radioimmunoassays and fluorescent antibody assay.

Major Results:

(A) The development of a test system. The most appropriate test system for evaluating the fatty acid requirements for normal mammary tissue is one in which the cell types characteristic of ducts and alveoli are present but with adipocytes absent. This criteria is met by the preparations we have isolated. These consist of a sphere (alveoli) or tube (duct) of epithelial cells surrounded by an apposing outer coat of myoepithelial cells, the whole unit being circumscribed by an outer layer of type IV collagen and PAS-positive material characteristic of basement membrane glycoprotein. In culture the alveolar cells dissociate from the basement membrane and proliferate as a monolayer. The ductal cells only partially spread away from the basal lamina and do not divide as shown by autoradiography. In culture the cells produce α-lactalbumin (~ 40 ng/10^{6} cells /day), a protein specific for mammary cells. The tumor cell aggregates as isolated contain little or no basement membrane components. In culture they spread and reveal two cell types, a central, compact plack of epithelial cells and a halo of stellate cells around the periphery. Stained sections of DMBA tumors also show two cell types in close proximity, a central core of epithelial cells banded by what is probably myoepithelial cells. The stellate cells seen in culture may represent this cell type. Autoradiographic analysis indicates that the epithelial cells divide 8 times as fast as the stellate cells in culture.

(B) Hormone and fatty acid effects on normal and tumor cell growth. In culture medium supplemented with lipid-stripped serum neither the normal or tumor cells exhibited significant growth in the absence of hormones. With hormones but without fatty acids, the doubling time was 58 hours for normal cells and 89 hours for cells derived from tumors. Linoleic acid reduced the doubling time to 30 and 35 hours for normal and tumor cells, respectively. Dose response curves for the most abundant fatty acids of rat mammary gland indicated that both normal and tumor cell growth was stimulatable by unsaturated fatty acids while saturated fatty acids were inhibitory. Normal and tumor cells differed in the concentration optima for the various unsaturated fatty acids and in the particular fatty acid giving maximal stimulation. Normal cells responded best to linoleic or linolenic acid while oleic acid enhanced growth maximally with tumor cells. For normal cells the concentration optimum for the unsaturated fatty acids was directly proportional

to the abundance of that fatty acid in the gland. The stimulation of unsaturated fatty acids and the inhibition produced by saturated fatty acids was detectable as early as 1 hour after addition to the cultures as reflected by the changes in ^{3}H-thymidine incorporation.

(C) Interpretation of the results. The most likely explanation for the above effects is that initiation of cell growth by hormones in the absence of unsaturated fatty acids produces a growth which is rate limiting, probably because of defficient transport. This limiting step is further restricted when saturated fatty acids are incorporated into cellular membrane but overcome by incorporation of unsaturated fatty acids.

Significance for Cancer Research: NCP objective #1, Approach 3). Information regarding two potential areas of concern to the etiology of mammary cancer and its progression may be gained from the project. One is the role of dietary fat in the incidence of breast cancer generally and the second relates to the development of metastatic potential. The latter derives from the fact that many primary mammary tumors or hyperplastic nodules can only be transplanted successfully to certain types of adipose tissue. This may be due to a high requirement for fatty acids by non-metastatic but not metastatic cell growth.

Proposed course of research: The findings presented as well as those of other investigators point to a co-ordinated system of cell types and tissue elements comprising the mammary gland with hormones such as prolactin not only acting on the epithelial cells but on adipocyte cells in concert to provide fatty acids needed for gland growth development and function. The *in vitro* explant culture approach will be pursued to ask questions about the selectivity for uptake and release of particular fatty acids from adipose tissue and hormone effects on the process. The primary cell culture of ducts and alveoli and DMBA tumor cells will be employed to determine the mechanism of activation or inhibition of cell growth by fatty acids and to explore the feasibility of selective killing of tumor cells by fatty acid analogs. To evaluate the hypothesis that metastatic potential of mammary cancer relates to a loss of a high lipid requirement, generally or specifically, a comparison of fatty acid effects on the growth of metastatic and non-metastatic rat mammary tumor cells will be undertaken.

Publications :

(1) Kidwell, W.R., Wicha, M. and Smith, G.: Fatty acid requirements for a cell line established from a 7,12-DMBA induced rat mammary tumor. In Lippman, M. (Ed.) Conference on Hormones and Cancer. Cancer Research (supplement). In press.

(2) Monaco, M., Lippman, M., Knazek, R. and Kidwell, W.R.: Effects of vasopressin on growth and differentiation of a rat mammary tumor cell line in long term culture. In Lippman, M. (Ed.). Conference on Hormones and Cancer. Cancer Research (supplement). In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05219-07 LPP

PERIOD COVERED
October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

In vitro Simulation of Hormone-dependent Mammary Tumor Regression

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R.A. Knazek	Senior Investigator	LPP, NCI
OTHER:	P.M. Gullino	Chief	LPP, NCI
	S.C. Liu	Chemist	LPP, NCI
	P.N. VanGorder	Chemist	LPP, NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Cell Cycle Regulation Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Target tissues respond to hormones only if specific receptors are present on or within their cells. Alterations of the hormone-receptor axis may then modify the response of target tissues (i.e. hormonally-responsive mammary carcinoma) to various hormones and serve as a means for controlling the growth and function of such tissues. We have shown that growth hormone induces the prolactin receptor in murine liver by acting at a site distal to the induced receptor. By making mice deficient in essential fatty acids, both the intraperitoneal and mammary gland fat are markedly decreased. These deficient animals have lowered levels of prolactin receptors and are unable to induce the receptors by the aforementioned hormone injections. Prostaglandins have also been shown to be required for the maintenance and induction of the prolactin receptor in vivo. These studies indicate that specific fatty acids modulate the functionality of prolactin receptors, probably by altering prostaglandin synthesis. The response of target tissues to various hormones may therefore be modified by the type and quantity of fat within the diet.

Project Description:

Objectives: Reproduce and study in vitro, the hormone-dependent regression process of mammary tumors in vivo.

Methods Employed:

Snell dwarf mice are autosomal recessive mutants whose circulating levels of growth hormone and prolactin are much lower than their phenotypically normal litter mates. These animals, consequently, attain only 1/4 of the normal weight.

A radioreceptor assay for prolactin and growth hormone receptors is used to determine the ability of cell membranes to bind these polypeptide hormones. Minor modifications of this procedure allow binding studies to be performed on intact cells.

An assay for prostaglandin E and F has been set up consisting of organic solvent extraction, silicic acid column chromatography, and radioimmunoassay.

Fatty acid concentrations are determined by gas-liquid chromatography.

Major Findings: We have shown previously that the autosomally recessive mutant Snell dwarf mouse lacks hepatic prolactin receptors but that they can be rapidly induced by injections of either lactogenic (oPRL) or somatotrophic (bGH) preparations.

After these initial observations, we set about to determine the mechanism of how receptors are induced. Our first concern was that the bGH preparations obtained from the National Pituitary Agency were contaminated with bPRL (and vice versa); either the major or contaminating minor components could therefore, have been responsible for induction of the receptor. Subsequent purification of both bGH and bPRL preparations by PAGE revealed that both hormones induced detectable prolactin receptors when either hormones were injected separately every 8 hours for 2 days. However, while bPRL bound to the induced receptor, bGH did not. This, with the observation that bGH binding sites were unaltered in bGH-treated mice indicated that bGH induced the prolactin receptor by interacting with a site distal to the induced receptor, perhaps even at a different cell or tissue.

Cyclohexamide did not prevent the effectiveness of bGH treatment on the induction of receptor. Whereas it did prevent the synthesis of somatomedin-like material which was determined by the chick embryo fibroblast assay. It therefore appears that protein synthesis may not be required for receptor induction.

Since both bGH and bPRL are known to be lipolytic, the above effects might be a result of modification of the lipid bilayer. It was subsequently shown that incubating phospholipase A_2 with primary isolates of hepatocytes from normal mice would augment prolactin binding to the cells. This effect was a function of time, temperature, and enzyme concentration. Phospholipase A, which also removes the β fatty acid from phospholipids, behaved in a similar manner whereas phospholipase C, which hydrolyzes the α ester

linkage did not. Addition of free fatty acids or prostaglandins E and F did not mimic the effect whereas the addition of lysolecthicin, one of the other products of the PLA_2-phospholipid interaction, did increase prolactin binding by 40% . While both PLA_2 and bGH increased PRL binding, the effects were of different magnitudes and probably operating by different mechanisms. Modification of the lipid bilayer in vitro, therefore, can alter the PRL binding.

The possibility, though remained that prolactin and growth hormone induced the prolactin receptor by mobilizing lipid stores which subsequently altered the hepatic bilayer to expose a preexisting but non-functional receptor. To test this thesis, the lipid character of the store was changed by feeding C3H mice diets that contained no essential fatty acids (linoleic and linolenic acids). Studies from the 1930's indicated that such deficient animals lactated poorly and were less fertile. In addition, demographic surveys of the past 20 years have shown an increase in mammary carcinoma in countries having a high fat intake. These could be a result of modifications in the responsivity of the mammary gland to various stimuli, perhaps by subtle modification of the breast prolactin receptor activity. Two groups of C_3H mice were fed such a diet beginning at 4-6 weeks of age. Animals receiving the deficient diets gained weight at a slower rate than controls and were significantly depleted of both mammary fat pads and intraperitoneal fat stores within 2-3 months. At this point no significant differences were noted between deficient and control animals with regard to serum prolactin and progesterone, estrus cycle, uterine estrogen receptors or hepatic prolactin receptors. After 7 months on the diets, however, a 23-38% decrease in basal levels of prolactin receptors was noted in ♀ C_3H. In addition, while the prolactin receptors of control ♂ were elevated when injected with prolactin, deficient animals were unable to respond to such stimuli. It, therefore, appears that essential fatty acids are required for both the maintenance and induction of prolactin receptors in the livers of C_3H mice. This could conceivably be due to changes in membrane microviscosity resulting from altered lipid composition and/or modification of the prostaglandin cascade. GLC assay of the microsomal pellets showed the animals to be EFA deficient with concentrations of linoleic acid 12% of controls. Photofluorometric polarimetry of the microsomal preparations did not however, reveal any detectable changes in the microviscosity of the membranes. Purified plasma membranes and/or more sensitive techniques may be required to detect changes in viscosity.

Since the essential fatty acids are the precursors for prostaglandins E_2 and $F_{2\alpha}$ prostaglandins were considered as possibly involved in the maintenance and induction of prolactin receptors. To test this, graded doses of indomethicin (a prostaglandin synthetase inhibitor) were injected into ♂ and ♀ DW/? (C_3H) mice. A dose of 7 µg/gm every 4 hours reduced the number of receptor sites by 80% within 2 days of treatment. A time course revealed this dose to exert its effects within 12-20 hours after the start of treatment. Indomethicin was subsequently shown to also block the ability of exogenous prolactin to induce prolactin receptors.

Four month-old C_3H ♀ fed EFA deficient diets from the time of weaning exhibited delayed mammary gland maturation, glands from 70% of the deficient animals being composed of rudimentary ductal structures without significant

whereas 40% of the controls were immature. This experiment, when performed using animals 2 weeks older, however, did not show a significant difference between the deficient and control groups even though both were depleted of fat within the mammary glands.

Significance for Cancer Research: Demonstration that prolactin receptors are induced by growth hormone indicates that this hormone may play a central role in the responsiveness of mammary carcinoma to hormonal stimuli. The fact that growth hormone appears to accomplish this without binding to the induced receptor, implicates a distal trigger site and indicates the importance of cell-cell or tissue-tissue interactions in vivo. The requirement for essential fatty acids and prostaglandins to modulate the prolactin receptors indicates that the lipid stores play an important role in maintaining the responsivity of target tissues to circulating hormones. This implies that the mammary fat pad may provide biochemical as well as structural support for the mammary gland and thereby control the gland's response to hormonal stimuli. These studies support and explain, in part, the demographic correlation of mammary carcinoma with high dietary fat intake throughout the world.

Proposed Course of Project: Development of mammary glands will be studied in mice started on essential fatty acid deficient diets while in utero. The role of prostaglandins in the induction of the prolactin receptor will be studied both in vivo and in vitro.

Publictions:

1. Knazek, R.A., Liu, S. C., and Gullino, P. M.: Induction of lactogenic binding sites in the liver of the Snell dwarf mouse. Endocrinology 101: 50-58, 1977.

2. Knazek, R.A., Liu, S. C., Graeter, R. L., Wright, P. C., Mayer, J. R., Lewis, R. H., Gould, E. B., and Keller, J. A.: Growth hormone causes rapid induction of lactogenic receptor activity in the Snell dwarf mouse liver. Endocrinology (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08205-07 LPP

PERIOD COVERED
October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

Application of The Artificial Capillary Culture Technique to Polypeptide Hormone Production

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R.A. Knazek	Senior Investigator	LPP, NCI
OTHERS:	David S. Frankel	Chemist	LPP, NCI
	P.M. Gullino	Chief	LPP, NCI

COOPERATING UNITS (if any)

J. Wei, Chairman, Department of Chemical Engineering, M.I.T.

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Cell Cycle Regulation Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The artificial capillary culture technique provides a pericellular microenvironment that closely resembles the in vivo state. This may allow normal cells to maintain their differentiated functions for prolonged periods of time in vitro. Cells are maintained in a physiologic state without many of the artifactual conditions imposed by more standard methods of tissue culture. The technique is therefore, especially well suited for the culture of cells of endocrine origin. Response of the cells to various physiologic agents are being studied as well as the secretion of various hormones over long periods with the possible application to continuous production of hormones for laboratory and clinical uses.

The culture unit has been modified by braiding two separate capillary bundles together enabling one bundle to be operated at a higher pressure than the other. This induces a gentle bulk flow betwen bundles that simulates lymphatic drainage of in vivo tissues. Measurement of labeled compounds of various molecular weights thus passed through solid cell masses grown between the capillaries; mathematical analysis of these data showed that both the convection and diffusion modes of mass transport are important within solid tumors.

Project Description:

Objectives: Attempts are being made to develop a method that provides both large quantities of polypeptide hormones and a means for studying their synthesis and secretion in vitro. A technique is also being sought that will allow the physiology of solid tumors to be studied in vitro.

Methods Employed: A bundle of tube-shaped semi-permeable membranes perfused with tissue culture medium, simulates the in vivo capillary bed. Isolated cells injected into the extracapillary space receive nutrients and have cell products removed by diffusion through the capillary walls. In this nearly physiologic environment the cells form solid masses of tumor.

GH3 cells are established rat pituitary tumor cells that secrete growth hormone and prolactin. These cells were obtained from the American Type Culture Collection.

Radioimmunoassay for rat growth hormone was performed using the double antibody method provided by the National Pituitary Agency.

Major Findings: The standard artificial capillary culture unit which was developed in this laboratory, has previously been shown to provide a nutrient matrix which permits established cells to grow and form solid tumor masses in vitro. Secretory cells have retained their function in such a system for several months. Modification of the recirculating perfusion medium into a single pass made of operation has demonstrated its value in studying the response of cell masses to physiologic hormones and chemotherapeutic agents.

The standard unit has recently been modified to include the following features. Two separate bundles of artificial capillaries are braided in such a manner that the bodies of the bundles are in intimate contact with each other in a woven configuration while the ends of each bundle remain distinct. They are encased in a plastic shell having a "Y" at both ends that permits perfusion of each bundle separately. Labeled dextran, inulin, or sucrose were recirculated within one perfusion circuit at pressures ranging from 760 to 1100 mm Hg while the other bundle was perfused in a single pass mode operating at atmospheric pressure. Appearance of the labeled compounds was monitored in the effluent from the high pressure circuit. Mathematical analyses of the transient and steady state concentrations yielded estimates of diffusion and convection constants for the tumor being studied. The steady state permeation rate was shown to be proportional to $MW^{-.75}$ while analyses of the convection data showed the hydraulic conductivity of tumor tissue to be 3-6 x 10^{-12} cm^4/dyne·sec. These transport coefficients were then correlated with mathematical models which were developed to describe intercapillary mass transport in tumors.

Significance for Cancer Research and the Program of the Institute: The characteristics of the capillary culture unit enable rapid responses to both stimuli and inhibitors of cell function to be studied in a nearly physiologic in vitro environment. This work demonstrates that convection

as well as diffusion plays a role in the transport of various substances within tumors. These data, in conjunction with the mathematical model of transport within tumors may be of assistance in pharmacokinetic modeling procedures and aid in understanding and improving the delivery of chemotherapeutic agents to solid tumors.

Proposed Course of Research: Various cell types will be studied within the artificial capillary culture unit in conjunction with our efforts to study the growth and regression of hormonally-responsive tumors in vitro.

Publications:

1. Knazek, R.A.: Endocrine Cell Culture on Artificial Capillaries. Pancreatic Beta Cell Culture, Fifth Hoechst Workshop Conference, Kitzbuehl, Austria, 1976 pp. 29-33, Excerpta Medica, Amsterdam, The Netherlands, 1977.

2. Knazek, R.A.: Tissue Culture Using The Artificial Capillary System. AIChE Symposium Series 170, vol. 73, Ed: W.R. Schmeal, pp. 120-122, AIChE, N.Y., 1977.

3. Frankel, D.S.:"Mass Transfer by Convection and Diffusion in Dual Circuit Artificial Capillary Cell Culture", Ph.D. Thesis, Department of Chemical Engineering, University of Delaware, Newark, Delaware, 1977.

4. Gullino, P.M. and Knazek, R.A.: Tissue culture on artificial capillaries. In: Methods in Enzymology, Ed: W. Jakoby, Academic Press, New York (in press).

5. Knazek, R.A., Gullino, P.M., and Frankel, D.S.: Dual circuit, Woven Artificial Capillary Bundle for Cell Culture (U.S. Patent Pending #850-810) Nov. 11, 1977.

6. Knazek, R.A., and Gullino, P.M.: Semipermeable Envelope for Cell Migration in Artificial Capillary Culture (U.S. Patent Pending #892-853) Apr. 3, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08230-02 LPP

PERIOD COVERED
October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)
Hormonal Control of Mammalian Follicular Maturation and Oogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. G. Hillier	Visiting Fellow	RRB, NICHD
OTHER:	G. T. Ross	Deputy Director	CC
	A. J. Zeleznik	Visiting Fellow	Diabetes, NIAMDD
	A. S. Rabson	Director, DCBD	LP, NCI
	R. A. Knazek	Senior Investigator	LPP, NCI

COOPERATING UNITS (if any)
Laboratory of Pathology, DCBD, NCI
Reproduction Research Branch, NICHD
Diabetes Branch, NIAMDD

LAB/BRANCH
Laboratory of Pathophysiology

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Cell Cycle Regulation Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Granulosa cells obtained from estrogen-primed, hypophysectomized immature rats have been transformed with SV-40 virus and established as cell lines in vitro. After preliminary passage through nude mice, these cells were demonstrated to form both ascites and solid tumors in Sprague-Dawley female rats. Culture of the ascites fluid rapidly re-establishes the cell line in vitro. Growth of the tumor is accelerated by bilateral oophorectomy and retarded by hypophysectomy. This system may serve as an in vivo/in vitro model for the study of chemotherapeutic agents and understanding of the pathophysiology of ovarian carcinoma.

PHS-6040
(Rev. 10-76)

Project Description:

Methods: Granulosa cells were isolated from the pre-antral follicles of estrogen-treated hypophysectomized rat ovaries and transformed in vitro with SV-40.

Major Findings: Two cell types of differing morphology were established: an epithelioid variety and a poorly adherent, rounded type, similar in appearance to the original, non-transformed cell. The latter, poorly adherent cells were subsequently established in shaker suspension cultures, exhibiting a doubling time of 17 hours. When these cell suspensions were injected IP into previously irradiated ♀ nude mice (300 Rads), a solid tumor nodule formed at the site of injection and a hemorrhagic ascites with peritoneal and mesenteric studding appeared. Serial transplantation of the ascites fluid into intact nude mice and then irradiated (300 Rads) female Sprague-Dawley rats resulted an ascites tumor that is transplantable in intact SD rats every 1-2 weeks. IP injection of as few as 50,000 cells resulted in both ascites and solid tumor formation with the histologic appearance of adenocarcinoma. Injection of 10^7 cells IP yielded discernable tumor growth within 6 days in more than 75% of the recipients that progressed, killing the animals within 2-3 weeks. Up to 75 ml ascites fluid typically containing 10^7-10^8 tumor cells/ml was obtained from these animals and served as the source for inoculation into fresh hosts. Hormonal responsivity was demonstrated by comparing tumor growth in hypophysectomized ± oophorectomized with oophorectomized estrogen-treated and control animals. Oophorectomy consistently enhanced the onset of measurable tumor growth by at least 2 days with solid tumor dimensions in oophorectomized rats 1.5-2.7 times greater than in sham-operated controls. Estrogen replacement or hypophysectomy reversed the effects of oophorectomy on the latency of tumor development.

Although the tumor has been serially transplanted up to 17 times in rats, the second transplant generation has been frozen in the Mason Research Institute tumor bank for public use. Subsequent injection of these stored cells into irradiated female nude mice produced the same picture of ascites and solid tumors which were transplanted into non-irradiated female nude mice and subsequently intact S.D. rats.

The transformed granulosa cell line which fostered the tumor line has been shown to be without detectable steroidogenesis or FSH, LH, PRL, estrogen, or progesterone receptors. The cell do possess, however, both EGF and insulin binding sites.

Significance to Cancer Research: Study of normal ovarian responses to various physiologic stimuli may provide added insight into the development of ovarian carcinoma. Established hormonally-responsive ovarian carcinomata that can be studied both in vitro and in vivo will provide a powerful tool for the study of both the pathophysiology of ovarian carcinoma and pharmacokinetic responses to various chemotherapeutic agents.

Proposed Course of Research: Non-transformed granulosa cells will be assayed for prolactin receptors under the stimuli of FSH and GH treatments in vivo. Prostaglandin synthesis and steroidogenic response of such cells to various hormonal agents will be studied in vitro.

Publications:

1. Hillier, S. G., Knazek, R. A., and Ross, G. T.: Androgenic stimulation of progesterone production by granulosa cells from preantral ovarian follicles: Further in vitro studies using replicate cell cultures. Endocrinology 100: 1539-1549, 1977.

2. Ross, G. T., Hillier, S. G., Zeleznik, A. J. and Knazek, R. A.: Gonadotropin and steroid hormones interactions in ovarian follicle growth, atresia, and steroid hormone synthesis. In: Proceedings of the VIII Meeting of the International Study Group of Steroid Hormones, Research on Steroids, Rome, Italy, Academic Press, 1977 (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08212-04 LPP

PERIOD COVERED
October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

Synthesis, Utilization and Degradation of Messenger RNA in Human Lymphocytes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Shelby L. Berger	Research Chemist	LPP, NCI
Others:	Connie Birkenmeier	Chemist	LPP, NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Pathophysiology

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Cellular and Molecular Physiology

INSTITUTE AND LOCATION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A study of the metabolism of polyadenylated mRNA in resting and growing lymphocytes revealed the presence of both short-lived and stable molecules. The labile mRNAs do not participate in protein synthesis. Stable molecules are found bound to polysomes or free in the cytoplasm. This system will be used as a model with which to examine the mechanism by which specific mRNAs are selected by the cell for translation. Chemical differences between the labile and stable molecules and the free and bound mRNAs will be investigated. Further studies will encompass normal, resting and growing cells as well as cultured lines of lymphoid origin.

PHS-6040
(Rev. 10-76)

Project Description:

Major Findings: The study of RNA isolated from living cells depends upon reliable methods for extracting and purifying the desired species. Most of these techniques are aimed at segregating RNA from the protein component of the cell in order to prevent co-mingling of nucleases with RNA molecules that serve as substrates. Nevertheless, it has often been difficult to isolate intact RNA molecules from cell lysates because nucleases are plentiful and resistant to denaturing agents. When intact molecules have been obtained, they sometimes represent an unknown portion of the total number available. Many investigators have bypassed technical problems such as these by examining only cells that naturally have low levels of nucleases. Others, motivated by an interest in a specific biological system, have employed a series of increasingly more and more powerful denaturing agents, such as urea and guanidine hydrochloride, to obtain intact RNA, particularly mRNA, from nuclease-rich cells. Such approaches have been successful, yet they all have a flaw in common: it is impossible to investigate the very rich field of protein-nucleic acid interactions under these conditions. Since the study of the processes regulating transcription and translation requires preservation of complexes consisting of diverse molecules, noncovalently bound, it is clearly essential to inhibit nucleases by gentle means. I have accomplished this in the resting lymphocyte by the synthesis of a powerful ribonuclease inhibitor suitable for use during cell lysis and during subsequent purification of ribonucleoprotein particles.

The use of this inhibitor has permitted the extension of previous work on the nature of labile and stable mRNAs in resting and growing lymphocytes. That labile polyadenylated mRNA was not an artifact produced by degradation of hnRNA during cell fractionation had already been demonstrated kinetically. With Dr. Herbert Cooper, I showed that the half-life of poly(A)-bearing mRNA was under 1 hour whereas the half-life of hnRNA^{+}poly(A) was approximately 7 hours. However, further proof was necessary. I have now shown unequivocally that hnRNA does not leak out of the nucleus whether or not the nuclease inhibitor is employed. During a 10 min pulse, only tRNA enters the cytoplasm. This accounts for approximately 10% of the label incorporated into nonpoly(A)-bearing RNA in the intact cell. No polyadenylated molecules appear outside the nucleus despite the fact that such molecules abound inside the nucleus. Furthermore, under lysis conditions in which active nucleases are made virtually inactive, there are no large labeled hnRNA-sized molecules in the cytoplasm regardless of the pulse and or pulse and chase conditions.

The labile mRNA^{+}poly(A) must be construed as a legitimate component of the cytoplasm. It has both a poly(A) "tail" and a size distribution similar to that of bulk mRNA^{+}poly(A).

The interaction of both labile and stable mRNA^{+}poly(A) with the protein synthesizing apparatus of the cell was investigated. I have already shown that approximately half the stable mRNA was found on polysomes while the remainder was free in the cytoplasm. The labile mRNA was more difficult to

evaluate. After a short pulse approximately 70 to 80% of the radioactive cytoplasmic poly(A)-bearing molecules were short-lived, depending on the donor, and greater than 80% were free in the cytoplasm. With longer labeling periods or with a chase incubation, the percentage remaining free decreased. Thus the nonpolysome-bound newly synthesized molecules always outnumbered the labile molecules; whether or not all labile molecules were free remained to be determined.

Accumulation curves were generated in order to determine whether any of the free molecules was an obligatory precursor for the bound molecules. In such experiments radioactive tracers are administered continuously to cells and at intervals during the labeling period both free and polysome bound labeled mRNA^{+}poly(A) are assayed. Such curves clearly demonstrated that there was no precursor-product relationship between free and bound molecules. The bound molecules accumulated linearly, virtually from zero time, in a manner independent of the rate of labeling of free molecules. Accumulation into free molecules was biphasic with a rapid rise to near saturating levels in 4 to 6 h followed by a much slower rate of accumulation. The slower rate was approximately equal to the rate of production of labeled bound mRNA^{+}poly(A). Correcting for saturation of the pool of tracer itself, these kinetics are consistent with the existence of two types of molecules in the free complement: molecules produced in large numbers which must also turn over rapidly and molecules produced in much smaller quantitites. In a non-growing system such as the resting lymphocyte, the latter must be stable. These findings confirm the existence of free labile molecules and both free and bound stable mRNA^{+}poly(A).

The data have also been used to establish whether any subclass of the free mRNA^{+}poly(A) molecules is stored. If there are no dormant molecules, then the rate of accumulation of free and bound stable messages should be proportional to the total amount of each type present in the steady state. Conversely, a discrepancy would suggest that there is a pool of untranslated stable mRNA which is not in equilibrium with the translated variety. Our results indicate that no such discrepancy exists. Therefore, the putative pool of stored mRNA must be very small or completely absent. Such conclusions can be drawn directly, only if the size distribution of free and bound molecules is the same. In experiments using formamide gradients or urea gels, both of which are denaturing, the size range of both classes was identical. These results were completely dependent on the use of the powerful ribonuclease inhibitor.

The ability of both kinds of nonpolysome-bound mRNA^{+}poly(A) to direct protein synthesis was determined. Using a mixture of protein synthesis inhibitors which prevents steps subsequent to the formation of the 80 S initiation complex, it is possible to drive excess mRNA into polysomes in intact cells. By this means, it can be shown that at least 36 to 50% of free stable polyadenylated mRNAs were capable of supporting protein synthesis and that none of the labile molecules was competent. A series of controls and calculations revealed that competition of stable, unlabeled mRNA for initiation sites could not have prevented the labile molecules from binding.

The function of the labile mRNA$^+$poly(A) was investigated. The possibility was considered that labile molecules were unnecessary for the activities of the resting cell, but essential for one or more early steps in lymphocyte activation. Such a theory demands that labile molecules made in resting cells be preserved long enough to be translated in growing cells. Since previous experiments showed that labile mRNA is absent from growing cells, such a mechanism should result in either stabilization of labile molecules or a prolongation of their half-life. When appropriate experiments were performed with phytohemagglutinin to activate the cells, the amount of mRNA$^+$poly(A) and its half-life in growing cells were identical with the same parameters measured in resting cells. Since these experiments must be performed immediately after stimulation of the cells and since mitogen treatment produces a highly asynchronous response, the experiments were repeated with secondarily resting cells. Such cells are transformed rapidly and more synchronously by mitogens. Nevertheless, indistinguishable results were obtained for both resting, newly stimulated and restimulated growing cells. As a result I have tentatively concluded that labile mRNA$^+$poly(A) is not unique but represents, instead, an overproduction of mRNA in the resting state which is corrected after growth stimulation. If this is the case, the phenomenon might be termed mRNA wastage in analogy with rRNA wastage already described by Cooper in resting lymphocytes.

Significance to Biomedical Research and the Program of the Institute: This project conforms to Objective #3, Approach #1 of the National Cancer Plan. It aims at an understanding of the biochemical control mechanisms by which normal cell growth and function are maintained. The causes of disordered cell growth and activity in neoplasia may then be better understood and rational attempts made to prevent or modify them.

Proposed Course: The differences between the labile and stable mRNAs will be investigated by chemical means since these molecules can now be prepared in intact form either as naked RNAs or as ribonucleoprotein particles. Sequence differences between free and bound stable mRNA, stable and labile mRNA, and resting and growing mRNA will be studied using cDNA probes. The ability of all of the molecules to support protein synthesis in cell free systems will be examined. These experiments are feasible because we are now able to translate bulk resting cell polyadenylated RNA with the wheat germ system. The investigation is entirely dependent on the nuclease inhibitor I have synthesized. Experiments aimed at understanding the mechanism of RNA turnover are also anticipated.

Publications:

1. Berger, S.L. and Cooper, H.L.: The relationship between hnRNA$^+$poly(A) in non-dividing human lymphocytes: Evidence for distinct synthetic pathways for mRNA precursor and non-precursor hnRNA. Biochim. Biophys. Acta, 517: 84-98, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08210-04 LPP

PERIOD COVERED
October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

Biochemical Changes Related to Onset and Cessation of Normal Cell Growth

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Herbert L. Cooper	Head, Cellular and Molecular Physiology Section	LPP, NCI
Others:	Klaus Resch	Visiting Scientist	LPP, NCI
	Richard Braverman	Chemist	LPP, NCI
	Lance Tibbetts	Research Associate	LPP, NCI
	Christiane Kruger	Visiting Fellow	LPP, NCI
	Theodore Wood	Chemist	LPP, NCI

COOPERATING UNITS (if any)

Laboratory of Biochemistry, Enzyme Chemistry Section, NIDR.

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Cellular and Molecular Physiology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To characterize the behavior of ribosomal particles in a non-growing cell population in which the rate of protein synthesis is maintained at low levels; to correlate such behavior with the limitation in protein synthesis so as to understand the mode of translational control employed by the cell in adjusting protein synthesis to growth and metabolic activities; to determine the regulatory steps which are operative in the enhancement of protein synthesis associated with the onset of cell growth; to relate modifications of the cell membrane with biochemical alterations regulating nuclear and cytoplasmic functions as they participate in control of cell proliferation; to detect and characterize the biochemical steps which control protein synthesis during the physiological cessation of cell growth and division.

PHS-6040
(Rev. 10-76)

Project Description:

Methods: Purification of lymphocytes from human peripheral blood by combined nylon column adsorption and density sedimentation. Preparation of cytoplasmic extracts for density gradient analysis of subcellular particle distributions. Extraction and purification of RNA from intact cells and from cell fractions, analyzed by density gradient, electrophoretic, gel filtration and chromatographic techniques. Preparation of subcellular components such as purified cell membrane (plasma membrane), ribosomal particles and subunits, soluble and particle-bound factors essential to protein synthesis, intact nuclei capable of supporting RNA synthesis, from lymphocytes and other cell sources, using a variety of techniques, such as nitrogen cavitation, detergent lysis, density gradient centrifugation. Cell-free protein synthesis using preparations from rabbit reticulocytes, lymphocytes, wheat germ. Chromatographic and cell fractionation techniques for separation and purification of cell proteins (dissociation factor). Direct and indirect assay techniques for determining dissociation factor activity in lymphocytes. Quantitation of specific tRNA levels by measurement of aminoacylation with radioactive amino acids. Preparation of enucleated cells (cytoplasts) by treatment with cytochalasin-B followed by density-gradient sedimentation. Other general laboratory, cell culture and radiolabeling techniques.

Major Findings: Detailed studies have been carried out on the effect of actinomycin-D on protein synthesis in resting lymphocytes and during growth stimulation. These studies originate in our earlier work which showed that the utilization of ribosomes, and hence the rate of protein synthesis, was maintained at low levels in resting lymphocytes by a rate-limiting step at initiation of translation. Actinomycin-D is well known to cause progressive decrease in protein synthesis and disaggregation of polysomes in eukaryotic cells. This was confirmed for lymphocytes, and it was shown that the effect is mediated through an action of actinomycin on the nucleus rather than any direct interference with protein synthesis. Since the only known nuclear action of actinomycin-D is interference with RNA synthesis and possibly of transport of RNA to the cytoplasm, it may be concluded that actinomycin interferes with protein synthesis by abolishing the supply of some RNA form.

Examination of the kinetics of the actinomycin effect showed that protein synthesis declined gradually in resting lymphocytes. However, the ability of protein synthesis to be stimulated by mitogens was lost very rapidly. This type of effect would be found if the rate of protein synthesis were regulated in resting cells by the presence of limiting concentrations of some RNA form. Interrupting synthesis of this RNA would cause protein synthesis to fall in a fashion related to the decay of the RNA, which might be moderately slow. However, inability to raise the concentration of the regulatory RNA in the presence of actinomycin would immediately abolish the capacity for stimulation of protein synthesis of the levels already occurring in the resting cell. The hypothesis that some RNA form is the rate-limiting component in protein synthesis in resting lymphocytes was tested. It was found that in resting lymphocytes there was no delay between addition of actinomycin and the decline

in the rate of protein synthesis. This indicates that the necessary RNA is already at limiting levels in resting lymphocytes. However, when lymphocytes were stimulated with phytohemagglutinin for 6 hrs. prior to administering actinomycin, there was a delay of 50 minutes before the rate of protein synthesis declined. This demonstrates that, following growth stimulation, RNA concentration is no longer rate-limiting for protein synthesis. When lymphocytes were allowed to grow for 24 to 40 hrs., the delayed effect of actinomycin was no longer seen. We interpret these findings to indicate that the rate of protein synthesis is regulated in resting lymphocytes by the concentration of some RNA form with relatively short half-life. Upon growth stimulation, an early event is an increased production of this RNA, which relieves the rate-limitation, permitting flow of ribosomes into polysomes and consequent rise in protein synthesis. As growth progresses, the large numbers of active ribosomes in growing cells again exceed the level of the regulatory RNA, and it once again becomes rate-limiting, albeit at a higher level.

Blocking protein synthesis with the drugs, cycloheximide and pactamycin allows the continued formation and accumulation ("trapping") of 80S initiation complexes. Addition of actinomycin-D abolishes this formation, demonstrating that the site of action of the regulatory RNA is at some step in the formation of the 80S initiation complex. Further studies are in progress to localize this site more precisely.

The nature of the regulatory RNA is under investigation. Messenger RNA and ribosomal RNA can be eliminated on the basis of prior knowledge, since both are highly stable in resting lymphocytes, and present in abundance. Transfer RNA (bulk) was found to have a half-life of >8 hrs. in actinomycin-treated resting lymphocytes, while the rate of protein synthesis fell 50% in 100 min.; $tRNA_i^{met}$, the most likely candidate, showed no change in amino acid accepting capacity during several hours of drug treatment. Thus, tRNA can be eliminated as the rate-limiting RNA. Ribosomal 5S RNA, a ribosomal RNA whose function is unknown, but which is transcribed independently of the major rRNA species, had a half-life of >5 hrs. in actinomycin-treated cells, making it, also, an unlikely candidate. Unexpectedly, it was found the bulk of 5S RNA was free in the cell sap, unassociated with ribosomes. The significance of this finding is unknown. We are currently investigating a variety of small RNAs of unknown function, found in the cytoplasm of lymphocytes and other animal cells. These appear promising, as they appear to be relatively short-lived. We are preparing bulk quantities of these species from rabbit liver, and they are to be tested for their activity in the rabbit reticulocyte cell-free protein synthesizing system.

Components of the reticulocyte system are also being used to examine other regulatory phenomena related to the low rate of protein synthesis in resting lymphocytes. The first step in protein synthesis, binding of met-$tRNA_i^{met}$ to 40S subunits in the presence of GTP and initiation factors, can be studied in this system. Preliminary experiments suggest that lymphocyte cytoplasm (post-ribosomal superatant) contains an inhibitor of this step.

The nature of this inhibitor, its role if any in regulation during growth stimulation, and its possible relationship to the regulatory RNA discussed above, are under investigation.

Previous studies revealed that an important permissive step in activation of lymphocyte protein synthesis was the mobilization of large numbers of inactive free ribosomes to enter polysomes. A required first step in this activation is the dissociation of 80S free ribosomes to 40S and 60S native subunits. This dissociation is mediated and stabilized by a protein complex called dissociation factor (DF). We have been adapting an assay for DF in lymphocytes under various conditions of growth, based on the ability of dissociated 60S ribosomal subunits to perform the peptidyl transferase reaction in the presence of N-blocked amino acids and 30% alcohol. This is still in progress. Unexpected difficulties have arisen, since it was found the 80S complexes also have some peptidyl transferase activity, complicating the assay. Also being developed is a radioisotope competition assay for D.F., which may solve these problems.

Preliminary studies indicate the presence of D.F. in post ribosomal supernatant from lymphocytes. Addition of purified plasma membrane vesicles appears to inactivate this D.F. We are exploring the question of whether the early stages of lymphocyte stimulation involve activation of previously inactive D.F. by modification of D.F. interaction with membrane constituents. In this way it is hoped to bridge one of the major gaps in our understanding of lymphocyte growth stimulation: what is the mode by which the "signal" provided by binding of mitogen at the plasma membrane is transmitted to internal sites with consequent biochemical changes?

The role of microtubules in early and late events in lymphocyte activation was examined, using colchicine to interfere specifically with microtubular function. It was found that colchicine had modest effects on the early events in lymphocyte activation: mobilization of ribosomes into polysomes, increased protein synthesis, and increased RNA synthesis. However, major interference with these processes occurred after 20 hrs. of stimulation, when the DNA replication phase of cell growth was in progress. This suggests, contrary to other published data, that microtubular activity is not a critical component of the early stages of lymphocyte activation.

In a collaborative study with the Enzyme Chemistry Section, NIDR, the activity and role of transglutaminase in lymphocytes during growth stimulation is being studied. This enzyme activity rises markedly following mitogen treatment. Moreover, it appears to be associated with the cell membrane fraction. Since this enzyme is capable of producing covalent interchain links in proteins through glutamic acid residues, it may be significant in the crosslinking phenomena which have been postulated to be involved in mitogen activation of

lymphocytes. Moreover, this enzyme is capable of linking polyamines to proteins. Since polyamine metabolism is increased following mitogen treatment, and since no clear role for polyamines in intact cells has yet been elucidated, we are searching for evidence of possible covalent polyamine-protein linkages related to transglutaminase activation. This may lead to identification of specific proteins which are modified during lymphocyte activation.

Significance of Biomedical Research and the Program of the Institute: By the elucidation of the mechanisms which regulate protein synthesis in resting cells and during a physiological growth sequence, we hope to provide the basis for recognition and understanding of derangements in these controls which may characterize malignant transformation. This objective supports Objective 3-1 of the NCP.

Proposed Course: 1) Identify and purify the RNA species which regulates protein synthesis in resting lymphocytes. 2) Define the site and mode of action of this RNA. 3) Examine the mechanism by which the production of this RNA is increased following growth stimulation. 4) Continue development of assay for DF and correlations of DF levels with early growth-related changes in ribosome activity. 5) Continue studies of interaction of cell membrane components with intracellular constituents to define the nature of the lymphocyte activation process. 6) Confirm and characterize the putative inhibitor of translation initiation found in lymphocyte cytoplasm.

Publication:

1. Cooper, H.L.: Ribosome Utilization and Regulation of protein synthesis during lymphocyte activation. In: Regulatory Mechanisms in Lymphocyte Activation. D. Lucas, Ed. Academic Press, New York, 1977, pp. 94-113.

2. Cooper, H.L. and Braverman, R.: Free Ribosomes and Growth Stimulation in Human Peripheral Lymphocytes. I. Activation of Free Ribosomes as an essential event in growth induction. J. Cellular Physiol., 93: 213-226, 1977.

3. Cooper, H.L. and Braverman, R.: The mechanism by which actinomycin-D inhibits protein synthesis in animal cells. Nature, 269: 527-529, 1977.

4. Berger, S.L. and Cooper, H.L.: The relationship between hnRNA^{+}poly(A) in non-dividing human lymphocytes: Evidence for distinct synthetic pathways for mRNA precursor and non-precursor hnRNA. Biochim. Biophys. Acta, 517: 84-98, 1978.

5. Harms-Ringdahl, M. and Cooper, H.L.: Sequential Changes in Ribosomal Activity During the Activation and Cessation of Growth in Lymphocytes Stimulated with Concanavalin A. J. Cellular Physiol. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08225-03

PERIOD COVERED
October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)
Membrane of the Mammary Gland Structure and Dynamics under Normal and Pathological Conditions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. Pinto da Silva	Head, Membrane Biology Section	LPP, NCI
	A. Peixoto de Menezes	Visiting Fellow	LPP, NCI
OTHER:	C. Parkison	Chemist	LPP, NCI
	N. Dwyer	Biologist	LPP, NCI
	R. Fucci	Biologist	LPP, NCI

COOPERATING UNITS (if any)
Dr. Ian Mather, Department of Animal Science, University of Maryland College Park, Maryland

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Membrane Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.6	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Comparative morphology of the plasmic membranes from lactating tissues and mammary carcinomas a) Membrane events during milk fat secretion; b) transformation of endoplasmic reticulum and golgi membrane during formation of fat droplet; c) Structure transformation of the membrane envelope of the milk fat globule after secretion and during isolation procedures.

PHS-6040
(Rev. 10-76)

Project Description:

Methods: Tissue (lactating rat mammary gland, DMBA and NMU mammary carcinomas) and membrane preparations (milk fat globule membrane - MFGM) are fixed in glutaraldehyde, impregnated with glycerol, and rapidly frozen in the liquid phase of partially solidified Freon 22 at liquid nitrogen temperature. Specimens were either fractured and platinum-carbon replicated in a Balzers freeze-etch device or prepared for conventional thin sectioning. All specimens were observed by TEM in a JEOL 100C EM.

Major Findings: Membrane events during milk fat secretion were analyzed by freeze-fracture of the rat mammary gland. Two modes of milk fat secretion were observed: extrusion of fat droplets surrounded by a portion of the apical plasma membrane of the alveolar epithelial cells and, less frequently, release into the alveolar lumen of fat droplets contained in intracytoplasmic vacuoles. The extrusion process consists of two asynchronous events: clearing of membrane particles (probably including integral membrane proteins) and bulging of the apical plasma membrane. Most fat droplets are extruded with a bilayer membrane envelope (milk fat globule membrane) partially devoid of particles. The segregation of membrane particles may represent the onset of a process of structural degradation of the milk fat globule membrane.

Investigation of the intracellular aspects of milk fat droplet formation show that fat droplets are formed by apposition of endoplasmic reticulum cisternae. Preceding apposition, there is a clearing of membrane integral components from the corresponding areas of endoplasmic reticulum. A similar sequence of events seems to operate with Golgi membranes. The processes are essentially similar in normal cells and in cells of DMBA or NMU induced mammary carcinomas.

Observation of the membrane envelope of the milk fat globule was analysed in cream preparations, during isolation procedures and in milk fat globule membrane preparations. Freeze-fracture methods were used to investigate the structure and dynamics of this membrane and freeze-etch techniques to study its surface morphology. Our results show that the membrane coat of the fat globule is in a dynamic state in which there are regions which fuse with the fat droplet. Sealing of membranes was observed for the first time and observed to involve the joining together of membrane edges rich in integral membrane components, in a zipper-like mechanism.

Significance to Research and the Program of the Institute: There is considerable evidence of the interrelationships between the secretory process and the malignant transformations of the mammary epithelial cells. All these events are related with a considerable development of a system of cell membranes; the membrane envelope of the milk fat globules carries antigens common to the cell membrane of mammary carcinoma cells; there are common hormonal stimuli involved in the lactogenesis and in the malignant transformation. Cell membranes are also involved in other events related with tumor invasiveness and metastization.

Publications:

1. Peixoto de Menezes, A., and Pinto da Silva, P.: Freeze-fracture observations of the lactating rat mammary gland: membrane events during milk fat secretion. J. Cell Biol. 76: 767-778, 1978.

2. Peixoto de Menezes, A. and Pinto da Silva, P.: Crio-fracture da glandula mamaria en lactacao: Participacao e alteracoes estruturais da membrana acinar durante a secrecao de gotas lipidicas. Soc. Cienc. Med. Lisb., in press, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08232-01 LPP

PERIOD COVERED
October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)
Sendai Virus and Erythrocyte Membrane. Binding, Virus Erythrocyte Fusion and Virally Induced Inter-erythrocyte Fusion

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. Pinto da Silva	Head, Membrane Biology Section	LPP, NCI
OTHER:	K. Shimizu	Visiting Scientist	LID, NIAID
	N. Dwyer	Biologist	LPP, NCI
	C. Parkison	Chemist	LPP, NCI

COOPERATING UNITS (if any)
NIAID, Laboratory of Infectious Disease, Respiratory Virus Section

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Membrane Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	0.5	0.7

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The interactions of Sendai virus with human erythrocyte membranes were observed and interpreted. Sites of attachment of Sendai virus were located by freeze-etching techniques. The fusion of erythrocytes induced by Sendai virus originates formation of intercellular plasmodesmata and a polymorphic network of membrane tubules.

PHS-6040
(Rev. 10-76)

Project Description:

Methods: Sendai virus harvested from allantoic fluid and clarified by centrifugation. Virus is incubated with washed human erythrocytes and incubated for one hour at 40°C and for 1 to 60' at 37°C. Tissue is processed for freeze-fracture and freeze-etching.

Major Findings: The early interaction of Sendai virus with the erythrocyte involves binding of virus to glycophorin molecules intercalated into one erythrocyte membrane. Localization of viral binding sites was determined by freeze-etching experiments. Binding of the virus results in the formation of membrane indentations which disappear upon incubation at 37°C. Fusion of the virus consists of incorporating the viral membrane into the erythrocyte membrane. Analysis of the freeze-fracture morphology of human erythrocytes during Sendai virus-induced fusion indicates that it occurs in several spots within regions of close membrane proximity and results in formation of plasmodesmata which provide cytoplasmic continuity. Radial expansion of the plasmodesmata causes the formation of a polymorphic network of membrane tubules with a lumen continuous with the external space. Further expansion is probably made at the expense of this tubular network which eventually ruptures and is reabsorbed into the membrane and/or lost as membrane remnants within the cytoplasmic space. Our results are best explained assuming that the virus particles do not directly participate in intermembrane fusion. (Pinto da Silva, P., Shimizu, K. and Parkison, C.: Fusion of human erythrocytes by Sendai virus: freeze-fracture aspects. Submitted for publication); Pinto da Silva, P., Shimizu, K. and Parkison, C.: Fusion of human erythrocytes by Sendai virus, freeze-fracture aspects. Proc. 6th International Congress, Kyoto, 1978).

Significance to Cancer Research and the Program of the Institute: The outer surface of membranes contain most or all of the chemical groups that provide the characteristics for cells. Cell surfaces are directly responsible for many membrane interactions, including membrane fusion which mediates most secretory mechanisms as well as many viral infections of cells.

It is considered that the abnormal behavior of cancer cells is reflected to a large degree by altered characteristics of their cell surface and thus their plasma membrane. Therefore, studying the structure and function of biological membranes in general will give a better understanding of the basic biological processes of cancer cells.

Proposed Course of Research: Additional experiments will be made to clarify structural differences between hemolytic and non-hemolytic Sendai virus and to obtain information on the mode of fusion of the virus with the erythrocyte membrane.

Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08234-01 LPP

PERIOD COVERED
October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)
Freeze-fracture Observation of the Normal and Cancerous Prostate Gland

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Pedro Pinto da Silva	Head, Membrane Biology Section	LPP, NCI
Others:	Robert Fucci	Biologist	LPP, NCI
	Clifford Parkison	Chemist	LPP, NCI
	Nancy Dwyer	Biologist	LPP, NCI

COOPERATING UNITS (if any)
Dr. J. A. Serrano, Dept. of Research Oncology and Cell Biology, Sinai Hospital of Baltimore, Baltimore, Md., and Lab. of Electron Microscopy, Univ. of the Andes, Merida.

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Membrane Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.20	0.15	0.5

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Freeze-fracture analysis of the prostate gland reveals a complex system of membranes including secretory vesicles, multivesicular bodies and condensing vecuoles. Thermal phase transitions are observed within endoplasmic reticulum membranes suggesting remarkable fluidity at physiologic temperatures.

PHS-6040
(Rev. 10-76)

Project Description:

Methods: Rat prostate gland is dissected, fixed, impregnated in 30% glycerol and freeze-fractured. Platinum-carbon replicas are observed with the electron microscope.

Major Findings: Aggregation of intramembranes particles of the endoplasmic reticulum is induced by incubation at low temperature. Morphological information on the functional complexes, secretory processes, endoplasmic reticulum and golgi membranes as well as condensing vesicles and secondary lysosomes has been and is being collected to prepare a monograph on the freeze-fracture morphology on this gland. Induction of secretory activity with pilocarpine is made in order to increase the chances of observation of aspects of secretory events.

Significance to Cancer Research and the Program of the Institute: Prostate cancer is one of the most prevalent cancers in man. Considerable efforts have been devoted to the study of the structure at the electron microscopic level of normal and malignant prostatic tissues. Most previous studies have used conventional thin sectioning. We attempt for the first time, to study with freeze-fracture, the morphology and dynamics of the membranes of the normal prostate. This step will be followed by a comparative study of prostatic carcinoma in order to evaluate the similaritites and differences with their normal counterpart.

Proposed Course: The morphology of normal and cancerous prostatic tissues will be compared.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 CB 08233-01 LPP

PERIOD COVERED

October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

Freeze-fracture Studies of the Schwann Cell Membrane and of Schwann Cell-axolemma Interactions: Morphological Study of the Garfish Olfactory Nerve and Search for Possible Axon-axon Interactions.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Pedro Pinto da Silva	Head, Membrane Regulation Section	LPP, NCI
OTHERS:	A. Peixoto de Menezes	Visiting Fellow	LPP, NCI
	C. Parkison	Chemist	LPP, NCI

COOPERATING UNITS (if any)

Dr. Rodman Miller, The Salk Institute for Biological Studies, La Jolla, CA

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Membrane Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.6	0.4	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Schwann cell membranes from the myelin sheaths of rat optic and sciatic nerves are observed during differentiation. Freeze-fracture analysis is used to determine the processes of myelin formation. Hexagonal particle rosettes are observed at the periaxonal regions of the Schwann cell plasma membrane in regional correspondence with particle clusters in the axolemma.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To study the mechanism of formation of myelin and the structural basis for possible functional interactions between the cytoplasm of Schwann cells and the axoplasm. To observe the axolemma of non myelinated axons and search for possible cytoplasmic communication between neighboring axons.

Methods Employed: Rat sciatic and optic nerves are excised, fixed and freeze-fractured. Age of animals 1, 3, 7, 15 days. Garfish olfactory nerves are dissected, fixed and freeze-fractured. Replicas are observed with the electron microscope.

Major Findings: Freeze-fracture observations confirm and extend previous thin section observations of the biogenesis of myelinated nerves. Hexagonal arrays (rosettes) of intramembranous particles are observed in regional correspondence with intramembraneous particle clusters within the axolemma. These results suggest the presence of intercellular communication between the Schwann cell and the axon. Analysis of the freeze-fracture morphology of the unmyelinated axons of the garfish olfactory nerve indicates fusion between axons and the presence of a linear light junction on the Schwann cell which envelopes the axon bundles (Miller, G. and Pinto da Silva, P. Freeze-fracture morphology of the garfish olfactory nerve; manuscript in preparation).

Significance to Cancer Research and the Program of the Institute: The investigation of structure and function of membrane is important to understand intercellular communication in normal and transformed cells and many other cellular processes at the level of the plasma membrane and chemistry of surfaces of normal and cancerous cells. Myelination can be a useful model to understand several aspects of the biogenesis of cell membranes. It is important to understand aspects of demyelination diseases and secondary nervous lesions in cancer and metabolic disease.

Proposed Course of Project: To finish the observations on the biogenesis of myelinated nerves. To prospect for the sites of initiation of Schwann cell membrane interactions which are observed in differentiated myelin. To examine the plasma membranes in the electric organ of the electric eel.

Publications:

1. Miller, R. G. and Pinto da Silva, P. (1977) Particle Rosettes in the Periaxonal Schwann Cell Membrane and Particle Clusters in the Axolemma Rat Sciatic Nerve. Brain Research 130: 135-141.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08235-01

PERIOD COVERED
October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)
Freeze-Fracture Observation of Platelet Membranes during Clot Formation.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Pedro Pinto da Silva Head, Membrane Biology Section LPP, NCI

COOPERATING UNITS (if any)
J. F. David-Ferreria, M. H. Miranda, Laboratory of Cell Biology, Gulbenkian Institute of Science, Oeiras.

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Membrane Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Freeze-etch electron microscopic observation of platelets during clot formation reveals aspects of the formation and growth of the fibrin network.

PHS-6040
(Rev. 10-76)

Project Description:

Methods: Human platelets are isolated and processed for freeze-fracture: i.e., fixed, impregnated in glycerol, frozen and freeze-fractured. In other samples the fracture of clots, previously induced, and the specimens processed in a similar way. After freeze-fracture the frozen fractured specimens are exposed to a long period of sublimation (deep freeze-etching). Platinum/carbon replicas are observed with the electron microscope.

Major Findings: Electron microscopic observation of human platelets during clot formation, after deep freeze-etching reveals, at the surface of their plasma membrane, initial aspects of the growth and formation of the fibrin network.

Significance to Biomedical Research and the Program of the Institute: This project is not primarily related to cancer. However, coagulation alterations are not unusual in cancer patients, although the exact link is still poorly understood. Some defects related to fibrinogen polymerization occur frequently in cancer patients and more information is needed to understand and control this paraneoplastic event.

Proposed Course of Project: To continue and complete observations on the formation of the fibrin network. To search for possible alterations in the structure of the platelet membrane which may be related to blood clot formation.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08236-01 LPP

PERIOD COVERED
October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

Membrane Fusion during Secretion of Peripheral Vesicles in P. palmivora Zoospores

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Pedro Pinto da Silva	Head, Membrane Biology Section	LPP, NCI
Others:	Donald Hemmes	Expert	LPP, NCI
	Clifford Parkison	Chemist	LPP, NCI
	Nancy Dwyer	Biologist	LPP, NCI

COOPERATING UNITS (if any)

University of Hawaii, Hilo, Hawaii

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Membrane Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.55	0.40	0.15

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Thin section observation of Pythophthora palmivora zoospores reveals the presence of electron dense deposits at the zones of membrane fusion.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To elucidate the role of calcium in the process of fusion of biological membranes; to understand other structural aspects of the mechanisms of membrane fusion.

Methods: P. palmivora zoopores are harvested, washed and induced to encyst under a variety of mechanical and ionic conditions. Encysting zoospores are fixed and prepared for thin section electron microscopy.

Major Findings: Electron dense deposits are observed at the regions of adjoining fusing membranes. Investigation of the chemical nature of the deposits indicates the involvement of calcium ions.

Significance to Cancer Research and the Program of the Institute: Membrane fusion is a frequent and important event in the biology of cells. It mediates not only most secretory processes but also other important phenomenen such as the formation of secondary lysosomes as well as endocytotic events. The study of plasma membranes, their structure, dynamics and interactions, is important to understand the biology of the normal cell and serve as a basis for comparison with pathological states including cancer.

Proposed Course: To investigate the nature of the putative calcium enriched electron dense deposits at the regions of membrane fusion.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00380-22 LPP

PERIOD COVERED
October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

Deoxyribose Containing Compounds in Normal and Cancer Tissues

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: W.C. Schneider Head, Nucleic Acids Section LPP, NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Nucleic Acids Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This program's objectives are to isolate and identify deoxyribose-containing compounds other than DNA from normal and tumor tissues, to determine the metabolic sequences in which these compounds are involved and whether these sequences are the same in normal, precancerous, and cancer tissues, to isolate DNA from subcellular structures other than nuclei, to study the physical and biochemical properties of extra-nuclear DNA and to determine the relationship between nuclear and extranuclear DNA in carcinogenesis.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: Since the replication of DNA is an obligatory prerequisite for cell division, the study of DNA synthesis and its control occupies a position of unique importance in cancer research due to the continuous and uncontrolled division occurring in cancer tissues. The objectives of this project are to isolate and identify deoxyribose-containing compounds other than DNA from normal, precancerous, and tumor tissues, to determine the metabolic sequences in which these compounds are involved and whether these sequences are the same in normal, precancerous, and cancer tissues; to isolate DNA from subcellular structures other than nuclei, and to study the physical and biochemical properties of extranuclear DNA and to determine the relationship between nuclear and extranuclear DNA in carcinogenesis.

These studies are aimed at uncovering differences between normal and malignant cells that can be exploited to prevent the formation of cancer cells or to promote the destruction of cancer cells already generated. (These studies support most closely Objective 3: To prevent conversion of cells to those capable of forming cancer and Approach 1: Nature and modification of the precancerous state, of the National Cancer Plan).

Methods: Rat liver cells were disrupted in suitable media using either commercial Potter-Elvehjem homogenizers with Teflon pestles or the Tekmar Tissumizer homogenizer. The homogenates were centrifuged at low speed to remove nuclei and unbroken cells and the sediments were rehomogenized with additional medium and recentrifuged. Mitochondria and microsomes were isolated from the combined supernatants by high speed differential centrifugation.

Yields of mitochondria and microsomes were calculated from the recoveries of the marker enzymes, succinic dehydrogenase for mitochondria and glucose-6-phosphatase for microsomes. Nucleic acids were precipated with the detergent CTAB (cetyl trimethylammonium bromide) and were extracted from the precipitates with 1.5 N perchloric acid at 70°. DNA was measured using the fluorimetric reaction with diaminobenzoic acid (DABA).

Major Findings: Several new approaches, described last year, to help in differentiating different types of cytoplasmic DNA have been unsuccessful and have been abandoned or held in abeyance. The use of restriction nucleases to characterize DNA samples failed to give consistent results with microsomal DNA - bands were obtained in preliminary experiments but could not be duplicated in subsequent preparations and the reasons for the failures could not be determined. Attempts to use cytoplasts (enucleated cells) in these studies also failed. This was due to the fact that the cytoplasts could not be disrupted to isolate the mitochondria and microsomes they contained. This problem has not been resolved. Finally, experiments designed to determine whether mitochondrial and microsomal DNA samples contained viral sequences already shown to be present in nuclear DNA were not completed.

Rewarding results have however been obtained in a new line of research measurement of DNA at very low concentrations and a reexamination of tissue cell disruption and fractionation.

DNA measurement. In order to measure DNA in cytoplasmic fractions, a very sensitive method had to be available. This was supplied by the fluorimetric diaminobenzoic acid (DABA) reaction of Kissane and Robins. Amounts of DNA in the 0.1 - 1.0 ug range could easily be measured with this method using pure samples of DNA. However, application to cytoplasmic samples of rat liver proved more difficult. Since these samples contained enormous concentrations of protein relative to the amounts of DNA present, the final reaction mixtures always contained large amounts of insoluble precipitates that interfered with the fluorimetric measurements. Attempts to extract the DNA from such samples before conducting the DABA reaction were only partially successful because the extraction reagents produced large blanks.

A solution to the problem was obtained by using (CTAB) to precipitate the DNA from the tissue samples. This reagent was shown to be quantitative at these low levels in experiments using added ^{3}H-DNA protein. In addition it was shown that CTAB could be used preparatively to separate DNA from most other tissue components and thus eliminate the requirements of phenol extractions.

Tissue disruption and cell fractionation. Using the CTAB-DABA method for DNA analysis, various kinds of homogenizers and homogenizing media were tested on rat liver. With 0.25 M sucrose as the medium, 1.5-2.0 per cent of the total DNA was recovered in the initial cytoplasmic fraction; this value was independent of whether loose or tight Potter Elvehjem homogenizers or a Tissumizer homogenizer were used. If the nuclear sediments were rehomogenized in sucrose using the same instruments to increase the yield of mitochondria and microsomes, alarming amounts of DNA, indicating nuclear breakage, appeared in the supernatant upon resedimentation of the nuclei. Values of 10-20 per cent of the total DNA were obtained with the Tissumizer or tight commercial pestle type homogenizers. If, however, rehomogenization was carried out with 0.25 M sucrose containing 10 mM tris buffer, the amount of DNA recovered was only 1 per cent. These experiments indicated that protective components are present in the cytoplasm of the tissue capable of preventing nuclear disruption even under conditions of high shear.

Even lower levels of DNA were obtained in the cytoplasmic fractions when homogenates were made in 0.25 M sucrose - 2 mM Mg++ or in 0.145 M KCl. This was due either to poorer cell disruption or to aggregation of mitochondria and microsomes in these solutions, however. In KCl, for example, less than 5 per cent of the mitochondria were released although the yield of microsomal material was about the same. This medium therefore provides a simple method for making microsomal preparations. Such preparations contained about 1 per cent of the total liver DNA.

Using the above information, it has now been possible to obtain data on the total DNA content of mitochondria and microsomes. This is done by measuring the DNA content of the isolated organelles and correcting for the yields as measured by appropriate enzyme markers. Values of about 0.3 per cent for mitochondria and 3 per cent for microsomes have been obtained. These figures were considerably different from previous estimates indicating that mitochondria and microsomes each contained about 1 per cent of the total liver DNA.

Significance to Cancer Research and the Program of the Institute: The experiments on DNA measurement described are important contributions to basic methodology and should have wide application in assessing the validity of cell fractionation procedures. Although early cell fractionation studies indicated that essentially all of the DNA was recovered in the washed nuclear fraction, this was perhaps due to the insensitivity of the method for DNA. This is in marked contrast to some recent reports in the literature in which 5-10 per cent of the DNA appeared in each cytoplasmic fraction including the cytosol. The present results show that the total cytoplasmic DNA content is of the order of only 2-4 per cent and demonstrate how cell fractionation procedures can be effectively monitored for nuclear disruption.

Proposed Course of Research: The experiments described above will be brought to a conclusion and the results prepared for publication. The findings will be applied in designing future studies on cytoplasmic DNA.

Publications:

1. Schneider, W.C. and Smith, G.H.: On the isolation of zepmogen granules from rat pancreas by zonal ultracentrifugation and their nucleic acid content. Anal. Bioche. 80: 373-382, 1972.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08238-01 LPP

PERIOD COVERED
October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)
Studies on the Purification and Function of Melanin DNA

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Alec S. Liacouras Chemist LPP, NCI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Nuclear Acids Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The long-range objective of this project is to investigate the function and properties of melanin DNA in murine melanomas. The initial aim is to purify the various DNA's from the nuclear, mitochondria and melanin granules, and characterize these with respect to melting points, density in CsCl and sedimentation coefficients. Nucleic acid hybridization studies will be used to determine the degree of interrelatedness between species, particularly with respect to the melanin DNA. The function of this latter DNA species will then be elucidated.

PHS-6040
(Rev. 10-76)

Project Descriptions:

Major Findings: Previous investigations [Schneider, Shelton and Kuff; JNCI 55, 665 (1975)] have shown that a species of DNA, different from that of either the nucleus or mitochondria exists in the melanin granules of the cytoplasm; this DNA was also found to be actively incorporating C^{14}-labelled thymidine. It is interesting to note that tyrosinase is thought to be the only enzyme present in the granules. The questions which natural arise are 1) how closely related is this species to the others found in the cell and 2) what is its function. We are now in the process of finalizing the purification procedure for each of these DNA's. The source material for our studies is the B-16 melanoma grown subcutaneously from a single cell suspension in C57/blk mice. After sacrificing the animal, the tumor is removed, homogenized in ice-cold sucrose/$MgCl_2$ and taken through a series of washings which separate the nuclear fraction from that of the mitochondria and melanin granules. The mitochondrial band and melanin pellet are then isolated by isopycnic centrifugation; a sucrose gradient was rejected since it was ineffective in eliminating succinic dehydrogenase activity (mitochondrial marker) in the melanin pellet. The nuclei, mitochondria and melanin granules were washed, and solubilized in urea-SDS (MUP) solution, and sodium perchlorate was added to assist both in the degradation of nuclear proteins and to reduce stickiness encountered during the solubilization process. We are also experimenting with proteinase K as a protein digest and with chloroform-isoamyl alcohol for protein extraction as possible inclusions in the purification procedure. As a final step, the purified DNA is chromatographed off hydroxyapatite by a step elution process. Using a combination of these latter methods, a small amount of purified melanin DNA has already been obtained and will be used in future characterization studies. Plans are now being developed to obtain a radioactive product using [^{14}C] thymidine which can be used in our hybridization studies.

Studies on uridine-cytidine kinase: Kinetic studies have recently been completed which explored the competition between 5-azacytidine, a triazine analog of cytidine, and each of the two natural substrates, to delineate whether they are phosphorylated by a single enzyme at the same catalytic site, or whether the two phosphorylations are mediated by separate isoenzymes that purified together. The theory and the patterns obtained have been described by Cha (mol. Pharmarol. 4: 621, 1968). We have found the Km and Vmax for 5-azacytidine, determined by the Cleland Hyper program, were 7.85 mM and 0.93 µmoles phosphorylated/min/mg protein, respectively, while those for uridine were 0.15 mM and 2.0 µmoles and for cytidine, 0.063 mM and 0.99 µmoles. Thus, the Vmax for azacytidine was half that for uridine and equal to that for cytidine. When uridine and 5-azacytidine were studied in combination, and plotted as 1/combined velocity against 1/uridine concentration, at several fixed concentrations of 5-azacytidine, a family of curves was obtained with an intersection near 0.21 mM uridine. The pattern resembled that for uridine plus cytidine, so the data agree with the theoretical for two substrates competing at the same enzyme site. In contrast with these patterns, for alternate substrates with the same Vmax values, the resulting

curves of 1/v against 1/substrate concentration did not intersect, but rather merged toward the respective Vmax value for either substrate alone, again in agreement with the theoretical. Thus neither substrate dominates the reaction velocity of the alternate substrate. To eliminate the possibility of a prejudiced visual interpretation, the curves and kinetic data obtained as a result of the Hyper program were compared to a second set generated by the MLAB computer program. These were calculated by fitting the velocity and concentration data to the mathematical function given by Cha (Mol. Pharmacol.). The graphic facilities of MLAB were also used to plot the best fit curves for v versus [S] and for 1/v versus 1/[S], at each fixed concentration of the alternative substrates. The Km and Vmax kinetics constants obtained in this way for the natural substrates and the analog, and the graphic representations were in good agreement with those obtained by Hyper. Further studies with [^{14}C]cytidine and unlabelled 5-azacytidine confirmed that the analog competitively inhibited the phosphorylation of cytidine. The inhibition pattern thus eliminated the possibility that 5-azacytidine may also be reacting non-catalytically at a site on the enzyme separate from the active center. The Km for cytidine was determined to be 0.05 mM, in excellent agreement with earlier data. The Ki for 5-azacytidine, determined from a plot of slope against 5-azacytidine concentration was 5.0 mM; studies with 5-azacytidine alone also gave a Km of 5.0 mM. These values agree with those from the alternate substrate studies. This work is now in its final stages of manuscript preparation for publication.

Significance to Biomedical Research and the Program of the Institute: It is becoming more apparent that DNA is present not only in the nucleus, but is also ubiquitous in the cytosol. However, little is known about the actual biological function or purpose of these DNA's. It is important therefore to discover how these various species interact within the cell, and in particular, within the neoplasm, and more importantly, how they are being regulated.

Proposed Course: Investigations will be continued on the isolation and characterization of melanin DNA. These include 1) repeating the original study, 2) obtaining a more sensitive marker for hybridization studies between melanin DNA and the DNA samples from the nuclear and mitochondrial fractions and 3) determining the function of this DNA species in the granules.

We also plan to continue some studies, on a collaborative basis, with uridine-cytidine kinase. These include analyses of some newly synthesized tricyclic analogs of uridine to determine their effectiveness as substrates, or possibly, inhibitors for the enzyme.

Publications:

1. Liacouras, A.S. and Anderson, E.P.: Uridine-cytidine Kinase III. Competition between Uridine and Cytidine for a Single Enzyme. Molecular and Cellular Biochem. 17: 141-146, 1977.

Dr. Elizabeth Anderson collaborated in the past with Dr. Liacouras and the last papers resulting from this collaboration are:

1. Anderson, Elizabeth P.: Female hormones and cancer of the breast. In Epidemiological Evaluation of Drugs, Clin. Pharmacol. and Drug Epid., Vol. 1, Proc. International Symp. on Epidemiological Evaluation of Drugs, Milan, Italy, May, 1977, (Colombo, F., Shapiro, S., Slone, D., and Tognoni, G., Eds.) Elsevier, Amsterdam, 1977, pp. 185-192.

2. Anderson, Elizabeth P.: Uridine-cytidine kinase from a murine neoplasm. In Purine and Pyrimidine Metabolism, Methods in Enzymology (Jones, M.E. and Hoffee, P., Eds.), Vol. LI, Academic Press, New York, 1978, ch. 40, pp. 314-321.

3. Anderson, Elizabeth P.: UMP - CMP kinase from Tetrahymena pyriformis. In Purine and Pyrimidine Metabolism, Methods in Enzymology (Jones, M.E. and Hoffee, P., Eds.), Vol. LI, Academic Press, New York, 1978, ch. 42, pp. 331-337.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08228-02 LPP

PERIOD COVERED

October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

Control of Synthesis of Serum Albumin and the Metabolism of Albumin Messenger RNA(mRNAalb.) in Normal Liver and Hepatic Carcinoma.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Pran K. Chakrabartty	Visiting Fellow	LPP, NCI
OTHER:	Walter C. Schneider	Head, Nucleic Acid Section	LPP, NCI
	S. K. Chattopadhyay	Senior Staff Fellow	PO, NCI

COOPERATING UNITS (if any)

Pediatric Oncology Branch, NCI

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Nucleic Acids Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Objective: The long range objective of this project is to examine the regulatory events involved in the synthesis of serum albumin. The translational control and post-translational processing will receive specific attention and the role(s) of hormone(s) in the synthesis of this protein will be studied. The effects of various carcinogens in the synthesis and metabolism of albumin and its mRNA will be tested. The relationship between nuclear, extranuclear DNA and mRNAalb. will be sought.

PHS-6040
(Rev. 10-76)

Project Description:

Malignant cells undergo continuous uncontrolled cell division. DNA, mRNA and protein synthesis are prerequisite for this process to continue. Because of the complexity of eukaryotic DNA it is necessary to have specific biochemical markers to study the regulation(s) of these processes necessary for cell division. Regulation of mRNAalb. has been elected as a specific marker for this purpose. The objective of this work is to isolate mRNAalb. or its complementary DNA (cDNA) to probe the involvement of mRNAalb. in normal, precancerous and cancerous liver tissues.

Methods Employed:

1. Isolation of mRNA by affinity chromatography of total RNA.

2. Translation of mRNA in a wheat germ cell free system and idenfication of synthesized ablumin by a double antibody immunoprecipitation method.

3. Treatment of the animals with a liver carcinogen - thioacetamide (TAA) to cause an increased synthesis of albumin mRNA.

4. Partial purification of mRNAalb. by size fractionation.

5. Synthesis of cDNA using partially pure mRNAalb. template.

Major Findings: mRNA was isolated from livers of untreated control and TAA treated animals. The TAA treatment was found to be associated with an increase in the relative quantity of polyadenylic acid-containing RNA. The translational activity of RNAs from 4 days TAA treated animals increased 2.5 fold whereas the synthesis of albumin directed by mRNA disproportinately increased reached a level of 5-fold in the wheat germ cell free system.

By size fractionation on sucrose gradient mRNAalb. could be partially purified and a cDNA was generated using this as a template.

Molecular hybridization with labeled rat unique DNA with RNA from either control or treated rat liver showed about 15% of the unique DNA is transcribed in both the cases.

Significance to Cancer Research: By use of albumin cDNA probe the levels of mRNAalb. can be precisely measured in normal, precancerous and malignant liver. The validity of a theory that microsomal DNA may be "messenger" DNA can also be tested using the probe in molecular hybridization. The establishment of a biological role of microsomal DNA, if any, in normal and cancerous tissue will be important.

Proposed Course of Research:

1. Purification of albumin cDNA to quantitate the levels of mRNAalb. in normal, precancerous and cancerous tissue.

2. Measurement of diversity of mRNA in normal and cancerous tissue by the use of cDNA to total mRNA.

3. Molecular hybridization of albumin cDNA with liver microsomal DNA to assess the biological role of microsomal DNA as "messenger" DNA.

Publications:

1. Qasba, P. K. and Chakrabartty, P. K.: Purification and properties of two forms of rat α-lactalbumin. J. Biol. Chem. 4, 1167-1173, 1978.

2. Chakrabartty, P. K. and Schneider, W. C.: Increased activity of rat liver messenger RNA and of albumin messenger RNA modulated by thioacetamide. Cancer. Res. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00306-15 LPP

PERIOD COVERED
October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

Modification of Amino Acids in Search of Possible Anti-tumor Agents

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Theodore T. Otani Research Chemist LPP, NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Nucleic Acids Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To prepare new amino acids and amino acid analogs and their derivatives by organic chemical methods in search of compounds that destroy cancer cells.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To prepare chemical compounds which preferentially destroy cancer cells by mechanisms other than those of agents presently being used in cancer chemotherapy. This study, which began with our search of a simple chemical method for the synthesis of the β-hydroxyamino acids, serine and threonine, led to the preparation of a series of novel β-hydroxyamino acids and to the study of the biological behavior of these compounds. These studies showed that alteration of certain β-hydroxyamino acids by acylation "activated" some otherwise relatively metabolically inert compounds to inhibit the growth of microorganisms which had been selected for anti-tumor screens. These findings led to the study of other amino acids and amino acid analogs that are known to be anti-metabolites.

The immediate objectives of the project have been: (a) To prepare acyl derivatives of amino acids and amino acid analogs and to note if these compounds exhibit microbial growth-inhibitory capacity in a microbial anti-tumor screen. (b) To prepare other new amino acid analogs, which by their structural resemblance to natural amino acids may be potential anti-metabolic, and hence, growth-inhibitory agents. (c) To prepare derivatives of these amino acids containing organic functional groups which may potentiate the growth-inhibitory as well as increase the cell permeability capacity. (d) To test these new compounds for anti-tumor activity, first in microbial anti-tumor screening systems, and if found active, in mammalian systems.

Methods Employed: The β-hydroxyamino acids were prepared by methods developed in this laboratory. An appropriate aldehyde is condensed with glycine in the presence of catalytic amounts of cupric ions (in some cases with glycine-copper complex) in a basic medium under conditions that are determined empirically. Diastereomers were separated by partition column chromatography. Optical isomers were resolved by means of asymmetric enzymatic hydrolysis using hog renal acylase or bovine pancreatic carboxypeptidase. In most cases, acylation was accomplished by the Schotten-Baumann reaction. For the trifluoroacetyl derivatives, because of the high reactivity of trifluoroacetyl chloride with water, the acylation was performed under strictly anhydrous conditions. In the benzoylation procedure, contaminating benzoic acid was removed by fractional crystallization with carbon tetrachloride and recrystallization from a single sovent system. In some cases, precipitation of the product from an ethanolic solution with water was required. Microbiological assays were carried out as described for the standard riboflavin assay, but with sufficient quantity of the vitamin to give maximal growth.

Major Findings: (1) Because of the activity noted with N-chloroacetyl-β-hydroxy-D-norleucine, diastereomer B, the preparation of gram quantities of its comparable leucine derivative, β-hydroxyleucine, was attempted using our method, starting with iso-butyraldehyde and glycine in the presence of an equimolar level of cupric ions, in basic medium. The product isolated was determined to be a racemic mixture of diastereomers A and B in the proportion of 4:1, respectively. Hence, although appreciable quantities of diastereomer A was isolated, only a small amount of the diasteromer B was obtained.

(2) Several attempts were made to prepare (a) γ-chlorothreonine, a chloro analog of β-hydroxynorleucine, from chloroacetaldehyde and glycine, (b) γ,γ-dichloro-α-amino-n-butyric acid, a dichloro analog of leucine, from dichloroacetaldehyde and glycine, (c) β-hydroxy-allylglycine, a β-hydroxy analog of allyglycine (which in turn is an analog of cysteine), from acrolein and glycine, and (d) β-hydroxy-tryptophan, an analog of tryptophan, from indole-3-carboxyaldehyde and glycine. Although various molar ratios of the aldehye to glycine, and of glycine to cupric ions were attempted under various temperatures, which ranged from 0 degrees to reflux temperatures, and reaction duration of a few minutes to days, none of these showed any remarkable formation of the desired product as determined by paper chromatography.

(3) β-Hydroxynorvaline, a lower homolog of β-hydroxynorleucine, was prepared successfully by the condensation of n-propylaldehyde with glycine in equimolar levels of cupric ions. The product was isolated in good yield. Its purity was established by elemental analysis, paper chromatography in 5 different solvent systems, and Van Slyke gasometric nitrous acid determination of primary amino nitrogen.

(4) The preparation of N-trifluroacetyl derivatives of amino acids and amino acid analogs was extended to include six more compounds, making the total number of trifluoroacetyl derivatives prepared to 18 compounds. The purity of these compounds was established by (a) melting point determinations, (b) elemental analysis, (c) optical rotation determination, where applicable, and (d) Van Slyke nitrous acid determination.

(5) The trifluoroacetyl compounds were tested for growth-inhibitory activity using Lactobacillus casei 7469 in a riboflavin-supplemented riboflavin assay medium. Of the 18 trifluoroacetyl amino acids so tested, 5 showed moderate, but distinct, activity at 1 mg/ml, final concentration. The compounds showing activity were N-trifluoroacetyl derivatives of β-2-thienyl-DL-alanine, β-3-thienyl-DL-alanine, o-fluoro-DL-phenylalanine, m-fluoro-DL-phenylalanine, and p-fluoro-DL-phenylalanine.

(6) When equimolar concentrations of these "active" compounds were compared, it was noted that their inhibitory activity was within the range of about 20-25% at a final concentration of 4.47 μmol/ml.

(7) A comparison of the level of inhibition of these N-trifluoroacetyl-phenylalanine analogs with a chloroacetyl derivative exhibiting inhibition showed that the level of activity of the trifluoroacetyl derivatives was about that of the active chloroacetyl derivatives.

(8) Because of the observation that the active compounds were all analogs of phenylalanine, an attempt was made to note if the inhibitory activity caused by these acyl compounds could be reversed by adding various amounts of phenylalanine or of acetylphenylalanine. No reversal of the inhibition was noted even when the reversal agent was present in equimolar quantities as the inhibitor.

(9) Twenty three acyl amino acids containing highly lipophilic side chains were prepared. These include 10 N-lauroyl-, 3 N-palmitoyl-, 3 N-stearoyl-, 1 N-myristoyl-, 1 N-oleoyl-, 2 N-linoleoyl-, and 3 N-linolenolyl-amino acid and amino acid analogs. These compounds. however, were found to contain appreciable amounts of the fatty acid as contaminants, and, hence, further purification is required.

(10) Preliminary studies on the preparation of cholesterol derivatives, where cholesterol may serve as the carrier of the active agents, were carried out. Crude (a) cholesteryl chloroacetate, (b) choleic acid of DL norleucine, and (c) choleic acid of N-chloroacetyl-DL-norleucine have thus far been prepared. However, further purification is required before characterization of these products is undertaken.

(11) Preliminary attempts have been made to prepare intermediates in the synthesis of amino acid derivatives not containing carboxyl groups. For example, preliminary to the preparation of the acyl chlorides and ultimately of alcohols of amino acids, the ethyl esters of DL-allyglycine, of m-fluoro-DL-phenylalanine, and of valine have been prepared by the conventional method of esterification. The products require further purification and characterization before the next step of treatment with thionyl chloride or phosphorus pentachloride is undertaken.

(12) Because of the alteration in activity noted upon chloroacetylation of certain amino acids and amino acid analogs, chloroacetyl derivatives of amino acid derivatives now being used in cancer chemotherapy were prepared. The purity of these compounds, too, were ascertained by melting point determination, elemental analysis, Van Slyke gasometric nitrous acid determination of primary amino nitrogen, and optical rotation determination, where applicable.

(13) Fifteen N-benzoyl derivatives of amino acids and amino acid analogs were prepared in this laboratory and 16 N-benzoyl derivatives obtained commerically were purified extensively. The purity was established, as above, by melting point determinations, elemental analysis, etc.

(14) Much of the time of this report period was spent in the attempt to resolve a problem connected with the identification of growth-inhibitory principle present in a commercial preparation of N-chloroacetyl-L-tyrosine. This compound when tested for growth-inhibitory activity on the L. casei system, showed inhibition greater than that shown by 6-mercatopurine, an agent presently used in cancer chemotherapy. During this study, althrough strong inhibition was consistently noted earlier, very suddenly, the results became quite erratic, marked inhibition being noted one day, moderate, little, or none the next. Attempts were made to determine the cause of this inconsistency, and many parameters were investigated. These include, (a) the organism, (b) the water used, (c) the medium (d) the glassware, (e) the size of the inoculum, (f) the quantity and age of the vitamin used, (g) the constituents of the medium, such as the content of ferrous ions, and the level of pyridoxal, (h) the temperature and duration of incubation, and

(i) the autoclaving condition. It was finally determined that the cause of the inconsistency was due to the heterogeneity of the compound, apparently the inhibitor being present in small clumps and distributed unevenly throughout the samples causing different amounts of it to be removed with each weighing. The identification of the active principle is being continued. Thus far, it appears to be a substance that is adsorbed on Dowex 50W, but further studies are required.

Significance to Biomedical Research and the Program of the Institute: That amino acid metabolism may be a susceptible area of action in cancer therapy was demonstrated by the efficacy of asparaginase in certain tumors which required asparagine. Amino acid deprivation, either by the removal of the amino acid or by the substitution with an analog may be effective in inhibiting tumor growth.

The unnatural amino acids synthesized under this program are close structural analogs of essential natural amino acids, differing in some cases only in the presence of a single hydroxyl group adjacent to the α-amino group. Hence, β-hydroxynorleucine differs from norleucine only in the presence of a hydroxyl group in the β-position of the carbon chain of the amino acid. β-Hydroxynorleucine is a structural analog of methionine, differing from the latter in having a methylene group in place of the sulfur atom in the γ-position of the chain. Indeed, β-hydroxynorleucine, even as a mixture of four isomers, exhibited inhibitory action in two microbial systems. β-Hydroxymethoxinine and β-hydroxymethionine are even closer analogs of methionine than is β-hydroxyhomomethionine, the former differing from methionine in possessing an oxygen atom in place of the sulfur atom and in possessing a β-hydroxyl group, and the latter only possessing a β-hydroxyl group.

The chain-halogenated amino acids, similarly, are close structural analogs of essential amino acids. For example, γ-dichlorothreonine would be an analog of leucine, since the size of the chlorine atom is approximately that of the methyl group; also γ-trifluorothreonine would be a close analog of threonine since the atomic radius of the fluorine atom is essentially that of hydrogen atom.

The enhancement of activity of amino acid, and amino acid analogs upon acylation appears to be of importance in the cancer program in view of the fact that one serious drawback in cancer chemotherapy has been that of the inaccessibility of the cytotoxic agent to the cell. It is possible that alteration of the molecular charge by acylation, and the introduction of a cytotoxic group, in the form of the chloroacetyl radical, has provided a molecule that is more permeable to the cell and at the same time, once within the cell, capable of releasing the cytotoxic agents by the action of intracellular acylases. Objective 6, Approach 2.

Proposed Course: (1) Because of the notable inhibition observed with N-chloroacetyl-β-hydroxy-D-norleucine B, further attempts will be made to prepare the analogous isomer of β-hydroxyleucine, i.e., N-chloroacetyl-β-hydroxy-D-leucine B. A chemical method for the selective preparation of the

B-isomer of this compound will be used. It will involve treatment of β-isopropylacrylic acid with bromine in aqueous solution, and amination of the resulting bromo-acid.

(2) The N-fatty acid acylated amino acid and amino acid analogs which were obtained in less than pure state will be purified, and the pure N-fatty acid acylated amino acid will be tested for growth-inhibitory activity.

(3) The N-benzoyl amino acid will be tested for growth-inhibitory activity in microbial anti-tumor screen.

(4) The cholesteryl compounds, and the various choleic acids will be further purified, and then characterized. Preparations of other compounds of this group will be attempted. These compounds will also be tested for activity in the microbial test system.

(5) N-Chloroacetyl derivatives of other amino acid anti-tumor agents will be prepared.

(6) Attempts to isolate and identify the active principle of the N-chloroacetyl-L-tyrosine will be continued.

(7) The pure optical isomer of the trifluroacetyl derivatives that have been found to be growth-inhibitory will be isolated from the racemic mixtures by asymmetric hydrolysis of the corresponding anilides using papain.

Publications:

1. Otani, T.T. and Briley, M.R.: Effects of acylated amino acids and acylated amino acid analogs on microbial antitumor screen. J. Pharm. Sci. 520-526, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00944 16 LPP

PERIOD COVERED
October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

Total Metabolism of Cancer Cachexia

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Seoras D. Morrison	Research Physiologist	LPP, NCI
Others:	John D. Radcliffe	Visiting Fellow	LPP, NCI
	Martin B. Popp	Surgeon	SURG, NCI

COOPERATING UNITS (if any)

Surgical Metabolism Section, Surgery Branch, NCI

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Energy Metabolism Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.2	1.7	1.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project is directed towards identifying the causes of the nutritional depletion and general deterioration of the host, known as cancer cachexia, with a view to blocking or reversing these systemic effects of cancer so that the cancer patient could become more accessible to anti-cancer therapies. The approach to this is by investigation of total energy, water and other material exchanges, and of the physiological and behavioral control of food and water intake in normal and cancerous organisms.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: (a) Investigation of the patterns of heat production of rats with concomitant recording of feeding behavior and other activity with a view to finding how the energy expenditure, feeding and general behavior pattern are related to tumor growth, to finding the nature of the loads imposed by a tumor on its host, and to identifying the causes of cancer cachexia. (b) Investigation of relative changes in water and material exchanges in tissue compartments of the rat during imposed and induced changes in food and water intake and the relation of these to tumor induction and growth. (c) Examination of possible experimental methods of simulating in non-tumor-bearing rats the cachexia and decline of food intake induced by tumor growth. (d) Identification of functional sites and causes of breakdown of control of food intake during tumor growth. (e) Development of conceptual models of control of food and water intake and regulation of energy and water exchange and their interrelationships for normal animals and for the cancer cachectic process.

Methods Employed: The methods of indirect, total, long-term calorimetry, operant conditional responses, placement of electrolytic lesions in or stimulation of the central nervous system, continuous or programmed infusions into unrestrained animals, and methods of orthodox nutritional studies. Ballistic methods of recording and partitioning total motor activity. Computer methods of numerical analysis of serial records of tumor growth, continous records of gaseous exchange and of change in feeding and drinking patterns in relation to total energy exchange and tumor growth. Sprague-Dawley rats, Buffalo rats and Fischer rats are used in all animal experiments. Most tumor-bearing rats are inoculated with Walker 256 carcinoma, but other transplantable tumors are also used.

Major Findings: Work is continuing on the behavioral and metabolic origins of the decline of food intake that is largely responsible for the cachectic decay accompanying tumor growth. Some of the individual components of normal feeding control that are impaired or unimpaired by tumor growth have been established. Attention is now being directed to whether, when these individual components are loaded in combination, the effects on food intake are additive, antagonistic or synergistic. Preliminary results suggest that they may be synergistic.

Work has been started on the effect of dietary amino acid deficiency or imbalance on differential accretion of N by host and tumor. A study has also been started on the metabolic efficacy of total parenteral nutrition during tumor growth. Preliminary results indicate that the metabolic consequences of this procedure are complex and not necessarily beneficial.

Significance to Biomedical Research and the Program of the Institute: The findings on control of feeding in tumor growth are throwing light on the nature of cancer cachexia and should be utilizable in the development of effective methods for improving the nutritional condition of cancer patients. The section of the National Cancer Plan that the work most closely approximates is: Objective 6 (Develop the means to cure cancers and to retard the progress of cancers not cured). Approach 4 (Enhance the host's ability to eliminate or prevent further development of Cancer). It is also immediately relevant to the 1974 Amendment to the Cancer Act (Collect ..... information respecting nutrition programs for cancer patients and the relationship between nutrition and cancer).

Proposed Course: Work in energy and water exchange of tumor bearers will be continued along with study on the interaction of metabolic and behavioral responses of normal and cancerous animals, with particular reference to the reasons for the changes in food and water intake and depletion of host tissues that occur during tumor growth. Objective 6, Approach 4.

Publications:

1. Morrison, S.D.: Extra-hypothalamic control of feeding. In Vigersky, R.A. (Ed.): Anorexia Nervosa. New York, Raven Press, 1977, pp. 75-79.

2. Morrison, S.D.: Origins of anorexia in neoplastic disease. Am. J. Clin. Nutr. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00941-22 LPP

PERIOD COVERED
October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)
Immunogenetic and Other Factors Affecting Bone Marrow Transplantation in Irradiated Mice

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: D. E. Uphoff Research Biologist LPP, NCI

COOPERATING UNITS (if any)

Radiation Physics and Computer Automation Section, ROB

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Radiation Biology Section

INSTITUTE AND LOCATION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Bone marrow transplantation in lethally irradiated mice and irradiated control mice were used to investigate the effects of qualitative differences in irradiation as they effect survival and the reproducibility of experimental data in this biological test system. The direction of the irradiation was an important factor with ventral exposure the least effective with dorsal exposure being less effective than the dual exposure from opposing X ray tubes. Qualitative differences in the radiation were apparently responsible for the development of partial chimeras and the failure to effectively eliminate preleukemic cells even in young mice. The need for more precise information on the irradiation facilities used in experimentation to be included in published reports is discussed.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To facilitate bone marrow transplantation as an adjunct to administration of toxic levels of irradiation or chemotherapeutic agents used in treating cancer. The ultimate goal is to devise methods of preventing a graft-versus-host reaction (GVHR) or controlling the lethal effects of a strong reaction while utilizing possible benificial effects of less severe reactions for their immunotherapautic effects against residual cancer cells.

Methods Employed: Multiple inbred strains and substrains of mice representing the major histocompatibility genotype (H-2), reciprocal F_1 hybrids homozygous and heterozygous for their H-2 alleles, and specially derived substrains are bred and maintained as a closed colony. Mice are exposed to lethal total body X irradiation and engrafted with marrow cells from different donors to test effects of genetic disparity between histocompatibility genes and immune response genes, as well as sex and parity status on the severity of the GVHR.

Major Findings: With the variety of genetic material available it has been possible to study the interrelationship between subtle differences in expression of tissue antigens of the irradiated recipient and the innate ability of the transplanted marrow to recognize and respond to these antigens. Although various methods of preventing and treating the GVHR have been demonstrated, no method has been devised that is universally applicable to all experimental conditions. This fact indicates that there are other unrecognized critical factors that may be unique for this experimental system. Until recently only biological parameters were considered in these investigations since the physical conditions involved in the radiation were constant. With the replacement of the modified 250 kV Westinghouse X ray machine with dual opposing tubes by a similarly modified Philips X ray machine, the importance of physical factors of the radiation became apparent. The new X ray equipment was fitted with the identical 0.25 mn Cu and 0.55 Al filters previously used on the Westinghouse machine. Adjustments were made so that the radiation produced by the new equipment could be reduced to ½ by 0.90 mm Cu, which constituted the half value layer (HVL) for the Westinghouse X ray machine. Expressing the quality of the X ray beam in terms of HVL is a common practice since it is generally assumed that biological effects of X rays are independent of the quality of the radiation. There was no experimental basis for the assumption that the biological effect produced by the radiation from these two machines would differ even though the wave length (or energy) distribution of the two X ray beams might differ. Differences in the biological effect of the radiation produced by these two machines was readily demonstrable in experiments involving bone marrow transplantation suggesting a qualitative difference in the radiation produced by the two machines.

The only previous experience with a demonstrable change in the biological effect of radiation without a change in either the calibrated dose or the HVL occurred when the line voltage regulator which was not an integral part of the Westinghouse X ray machine was temperarily disconnected. The addition of a

line voltage regulator to the Philips equipment also changed the results obtained in some marrow transplantation experiments without altering either the calibrated dose or HVL. In evaluating the results of experiments performed using these different conditions of exposure to X rays, additional critical factors not previously considered important became apparent.

The simultanious use of the two opposing X ray tubes reduced the exposure time and insured a uniform tissue dose in large animals. After the installation of the Philips equipment there were several long periods when only one X ray tube was operational. The exposure time was doubled in the belief that, for small animals, the same results would be obtained as long as the total dose (measured in air) was constant. Experience with the Westinghouse equipment supported this assumption since there had been no discernible difference in results when mice were expose to X rays produced by the double-tube exposure in Building 10 and the single dorsal exposure administered by the single Westinghouse tube located in Bldg. 8. However, most of these comparisons were made at or near the lethal level where the difference between the double tube and dorsal exposures are less readily detected. The dose-response curves for exposure to X rays from the Westinghouse machine were steeper than those obtained using the Philips equipment (even after the extra line voltage regulator was attached to the Philips machine). In addition there was a clear indication that results obtained with both the dorsal and ventral exposures differed from those obtained with the dual exposure to the same total dose. This difference occurred with both radiation alone and with radiation followed by marrow transplantation. In all 8 inbred strains where sufficient data is available, ventral exposure to total doses between 650 R and 800 R results in a marked reduction in mortality compared with the double tube, two directional exposure to the same total dose. Dorsal exposure results in mortality curves intermediate between the 2 extremes. As the total dose is increased at 25 R increments the mortality curves for the dorsal exposures approach those of the 2 directional exposure. The dose range used is well below that expected to produce central nervous system damage. Therefore the difference in biological effect of dorsal vs ventral exposures probably reflects the importance of the effective total dose administered to blood forming areas of the skeleton on the dorsal side of the mouse and does not constitute a dose-rate effect since the time of exposure is identical. This effect is tentatively being attributed to the combined difference in quality of the radiation and the dose reduction resulting from absorption as the X rays pass through the mouse tissue. Under conditions of exposure the total dose calibrated in air would be expected to be reduced by approximately 10% as the X rays passes through each cm of mouse tissue. For an adult mouse 3 cm thick, the dose reduction of the ventral exposure could be as much as 120-170 R depending upon the lethal dose for the particular strain being irradiated. Irradiated mice survive when a sufficent number of marrow stem cells fail to receive an adequate total dose of radiation or are protected by lead shields. The same phenomenon would account for the mixed chimerism observed in some irradiated mice receiving marrow inoculations. To test this explanation a

mouse model is being designed by the Radiation Physics and Computer Automation Section of R.O.B. to test the dose reduction effects at different levels as well as under different conditions of exposure, i.e., double tube vs dorsal and ventral exposures both with and without the additional line voltage regulator.

These experiments involving the physical aspects of irradiation were undertaken after it was found that AKR leukemias were less effectively influenced by the radiation from the Philips equipment as compared to the Westinghouse equipment. Only when AKR mice were 5 months of age or older at the time of exposure to irradiation from the Westinghouse machine and inoculated with compatible allogeneic (foreign) marrow of the $H\text{-}2^{k}$ genotype did leukemias of AKR tissue origin occur. In this case the radiation failed to illuminate a radiation resistant population of preleukemic cells which persisted up to 6 months after the treatment of the older mice. After similar treatment with the Philips machine even young recipients developed leukemia of recipient origin which indicated survival of recipient stem cells resulting in mixed chimerism. Other treatments such as the use of AKR marrow preincubated with allogenic erythrocytes or bovine serum albumin to protect lethally irradiated AKR recipients were less effective in reducing the incidence of leukemia following the installation of the new X ray equipment than was the case in the initial report (JNCI 42, 243, 1969 and JNCI 44, 1099, 1970). With the failure to reproduce data that had previously been reproducible, the formal publication of all experiments involving irradiation with the Westinghouse X ray machine was postponed until the difference in biological effectiveness of the radiation was resolved. Despite the generally held belief that biological systems are not affected by the quality of the irradiation, all evidence supports the hypothesis that the failure to reproduce existing data reflects a qualitative difference in the radiation. Since the reproducibility of experimental data by others is essential for acceptance of new concepts, it is imparative that more precise information on the irradiation facilities used be included in published reports.

Emphasis has been placed on the source of the radiation as the key factor in the reproducibility of these experiments since the mice are still being raised within the laboratory and other conditions are constant. The first indication as to the possible effects of the radiation source came with disconnection of the line voltage regulator from the Westinghouse X ray machine in 1972. At that time one set of experiments was repeated using a cobalt-60 source to determine which mortality curve represented the "true picture". The use of the Westinghouse X ray machine with the voltage regulator and the Co-60 source resulted in identical mortality curves while repeated use of the Westinghouse machine without the voltage regulator and the Philips machine with and without the voltage regulator resulted in quite different mortality curves. It would appear that without the same X ray equipment one can hardly expect to reproduce data from experiments duplicated in another laboratory yet this is the test of their validity. The early experiments with AKR leukemias mentioned above that could not be reproduced with the new

X ray equipment were recently successfully repeated by Dr. Robert Trutt of the Winter Research Laboratory, Mount Sinai Medical Center in Milwaukee using a cesium-137 source for the radiation again demonstrating the reproducibility of data obtained by use of the Westinghouse equipment provided an adequate radiation source is available. The wave length spectrum of the Westinghouse machine is available for comparison with the Philips X ray machine. If the spectrum for the new machine were measure the problem of obtaining reproducible results would still exist. However, we would know what combined wave lengths are required to produce a desired effect. Another alternative is the use of a cesium-137 source for the irradiation. With a half-life of 30 years, the consistant quality of the radiation would be assured for a considerable length of time and insure the reproducibility of replicate experiments conducted in different laboratories. It is obvious that the use of the HVL of a 250 kV X ray machines of different engineering designs and manufactured of different target and port materials etc can no longer be considered an adequate measure of X rays used for biological research.

Significance of Biomedical Research and the Program of the Institute: Clinical marrow transplantation is being used in various Institutes throughout the world. This treatment has been most successful for treating deficiency diseases, where irradiation has not been a factor, provided suitable donors are available. Marrow replacement for leukemic patients, where irradiation is an integral part of the protocal, has been the least successful and still constitutes a last ditch effort to control the disease. From the recent experience with the effect of radiation equipment on the results obtained in the mouse model there appears to be important factors related to the treatment of leukemia with marrow replacement that have not been considered here-to-fore. The same X ray machine used in these experiments is also used by immunologist and other investigators throughout the National Institutes of Health to produce T and B cell deficient mice. It would seem logical to expect the same type of interference with this experimental system as has been encountered in marrow transplantion experiments. It is clear that X rays as a source of ionizing irradiation for biological experiments need to be characterized with greater specificity than has been done here-to-fore. A preferable alternative would be to have a cesium-137 source available for basic biological research.

Proposed Course: Characterization of the effects of physic factors involved in the irradiation of mice in preparation for marrow transplantation will continue. With the completion of the dose response studies animal space is being made available to resume the investigation of runt disease in suckling hybrid mice by determining whether the intra uterine environment or milk is responsive for this syndrome.

Publications:

1. Uphoff, D.E.: Runt disease in suckling hybrid mice: Substrain differences and litter seriation. Transplantation Proc. 9: 1455-1458, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00942-19 LPP

PERIOD COVERED
October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)
Effects of Gamma Irradiation on Nucleic Acids and Proteins: Interactions of Cancer Chemotherapy Agents and Radiation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. Riesz	Research Chemist	LPP, NCI
OTHERS:	S. Rustgi	Visiting Fellow	LPP, NCI
	A. Joshi	Visiting Fellow	LPP, NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Radiation Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.8	2.0	0.8

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The effects of ionizing and ultra-violet radiation on nucleic acids and proteins and their constituents are being studied. The modification of radiation damage in DNA by cancer chemotherapy agents of the intercalating and alkylating types is of interest, since such information may be useful in radiation therapy.

The present report includes electron spin resonance (E.S.R.) studies of spin-trapped radicals produced by the reaction of hydroxyl radicals and of hydrated electrons with constituents of proteins and nucleic acids.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: The effects of ionizing and ultraviolet radiation on biological macromolecules and their constituents are being investigated. For reproductive death, DNA is the target molecule in viruses and is at least a part of the target molecule in bacteria and mammalian cells. Radiation damage to DNA is produced by the "direct effect" through the formation of radical ions, electrons, excited states and neutral free radicals or by the "indirect effect" where radical species formed in the surrounding medium by radiation react with DNA. For water, these species are hydrated electrons, hydrogen atoms and hydroxyl radicals. In the case of radiation damage to the chromosome, radicals formed in the nucleohistone may cause damage to the DNA.

In the chain of events that leads to loss of biological activity, free radicals play an important role. Chemical compounds have been discovered which significantly modify radiation effects. These include: (a) electron affinity sensitizers which act on hypoxic tumor cells, (b) halogenated pyrimidines which are incorporated into DNA and (c) cancer chemotherapy agents of the intercalating or alkylating type which sensitize tumor and normal cells.

Studies of the mechanism of action of radio-sensitizers and radio-protectors are necessary to design improved combinations of chemotherapy and radiation therapy.

An understanding of the mechanisms by which ionizing radiation brings about the loss of biological activity in macromolecules is likely to help in the development of new methods for altering the efficiency of cell killing with possible benefits to radiation therapy.

Methods Employed : Nucleic acids, proteins and their constituents were γ-irradiated either in the solid state at 2×10^{-5} torr or in aqueous solutions in an 800-curie Cobalt γ-source. Electron spin resonance studies were carried out with a Varian E-9 ESR Spectrometer connected to a Nicolet Lab 80 computer. For photolysis studies at specified wavelengths, a 1000-watt high pressure Mercury-Xenon arc source and monochromator were employed.

Major Findings:

1. ESR and spin-trapping studies of the reactions of hydrated electrons with dipeptides (with S. Rustgi).

The reactions of hydrated electrons (e^-_{aq}) with 55 dipeptides and 25 acetyl and formyl amino acids have been studies by e.s.r. and spin-trapping techniques. Gamma-radiolysis of deaerated aqueous solutions was used to generate e^-_{aq} and Na-formate or t-BuOH was added to scavenge the OH radicals. t-Nitrosobutane was employed as the spin-trapping reagent. The radical, $-\dot{C}O^- - NH -$, which is the initial product of the reactions of e^-_{aq} with dipeptides was observed only for val-gly, val-ala, val-leu and ile-ala.

For most of the dipeptides this radical converts to the primary deamination radical, $\dot{C}HR' - CONH - CHR - COO^-$, where R and R' are the side chains of the common amino acids. In many cases a radical of the type, $\dot{C}HR - COO^-$, formed by secondary deamination was also observed. Only secondary deamination reactions were observed for dipeptides containing β-alanine as the amino terminal residue and for acetyl and formyl amino acids. The secondary deamination reactions of e^-_{aq} with dipeptides, acetyl and formyl amino acids in aqueous solutions have not been observed previously. This type of reaction is of interest since it brings about main chain scission in polypeptides and proteins.

2. Hydrated electron initiated main chain scission in peptides: An ESR and spin-trapping study (with S. Rustgi).

The reactions of hydrated electrons (e^-_{aq}) with nineteen tripeptides were investigated. Hydrated electrons were produced as described above (section 1). t-Butanol was also used to scavenge hydroxyl radicals. The short-lived radicals formed by the reactions of e^-_{aq} with the peptides were spin-trapped with t-nitrosobutane to form stable nitroxide radicals and identified by e.s.r. spectroscopy. The tripeptides studied contained two glycine residues. Following the addition of e^-_{aq} to tripeptides, C - N bond scission at three sites was observed. Cleavage occurred between the nitrogen of the ammonium group and the α-carbon and between the nitrogen of the peptide linkage and the adjoining α-carbons. The radicals corresponding to each of these three types of scission were identified. From a comparison of the radical yields of the reaction of e^-_{aq} with ala, $(ala)_2$, $(ala)_3$ and poly-DL-alanine, with an average degree of polymerization of 1800, it was shown that e^-_{aq} can react with many carbonyl groups of poly-DL-alanine leading to main chain scission. Analogous reactions of e^-_{aq} with proteins and enzymes may be expected to lead to loss of biological activity.

3. An ESR and spin-trapping study of the reactions of the $\dot{S}O_4^-$ radical with protein and nucleic acid constituents (with S. Rustgi).

Reactions of the $\dot{S}O_4^-$ radical, generated by UV photolysis of $Na_2S_2O_8$, were studied in aqueous solutions of amino acids, dipeptides, nucleic acid bases, nucleosides and nucleotides. The transient free radicals so formed were spin-trapped by t. nitrosobutane and identified by e.s.r. spectroscopy. The amino acids primarily undergo oxidative decarboxylation. The pK's of the ammonium group of the spin-trapped decarboxylated radicals of glycine and alanine in D_2O were determined to be 8.3 ± 0.2. An oxidation product, which is the precursor of the decarboxylated radical, is tentatively identified for alanine, valine and isoleucine. Radicals formed by H abstraction by $\dot{S}O_4^-$ are identified for leucine, serine, phenylalanine and 4-hydroxyproline. In dipeptides, $\dot{S}O_4^-$ produces decarboxylation of the amino acid located at the carboxylate terminal residue. For gly-ala and ala-ala, radicals generated by H abstraction from the carboxylate terminal residue alanine were also characterized. Radicals centered on the C(5) carbon were observed for uracil, cytosine and thymine. For nucleosides and nucleotides, radicals situated on the base and/or the sugar moiety were assigned.

4. Free radicals in UV irradiated aqueous solutions of substituted amides. (with S. Rustgi)

The radicals produced by reactions of hydroxyl radicals with alkyl substituted ureas and amides in aqueous solutions have been investigated. Hydroxyl radicals were produced by U.V. photolysis of H_2O_2 and the short-lived amide and urea radicals were spin-trapped by tert. nitrosobutane and identified by e.s.r. For all N-alkyl derivatives of urea and acetamide and for N,N-dimethyl propionamide and N,N-diethyl formamide only radicals centered on N-alkyl groups were detected. Radicals situated on only alkyl groups attached to the carbonyl carbon were observed for dimethyl acetamide, trimethyl acetamide and butyramide. However, for N,N-dimehtyl butyramide, N,N-diethyl butyramide, N-methyl propionamide and N,N-diethyl propionamide free radicals were formed which were localized on the alkyl group attached to the amide carbon as well as those attached to nitrogen. The H atom bound to the carbonyl carbon was abstracted in N-ethyl formamide. Acyl radicals formed by C-N scission due to direct U.V. photolysis of N,N-dimethyl butyramide and N,N-dimethyl propionamide were also detected.

5. E.S.R. of free radicals in aqueous solutions of substituted pyrimidines (with S. Rustgi).

Reactions of OH radicals with methyl and ethyl derivatives of uracil, cytosine and thymine in aqueous solutions have been investigated. Hydroxyl radicals were generated as described previously (section 4). Addition of OH radicals was found to take place predominantly to the C(5)-C(6) double bond of the bases. H-abstraction from the methyl group occurred in the N(1) methyl derivatives of uracil, cytosine and thymine. Radicals formed by H-abstraction from the methyl group were also detected for 3-methyluracil, thymine, 1-methylthymine and 1-ethylthymine. Introduction of a methyl or ethyl group at the N(1) position of uracil, cytosine and thymine causes an increase in the C(6) proton coupling and a decrease in the N(1) splitting for radicals formed by OH addition at the C(5) position.

6. E.S.R. of spin-trapped radicals in aqueous solutions of dihydropyrimidine bases (with S. Rustgi).

The reactions of hydroxyl radicals with several dihydropyrimidine base derivatives in aqueous solutions have been investigated by the technique of spin-trapping and ESR spectroscopy. Hydroxyl radicals were generated as described previously The dihydro derivatives of uracil, 6-methyluracil, 1-methyluracil, 1-ethyluracil, 1-methylcytosine, 5-methylcytosine, thymine, and orotic acid were investigated. Predominant reaction in all cases was the abstraction of the H atoms bound to the C(6) position. To a lesser extent H-abstraction took place from the methyl groups attached to the N(1), C(5) and C(6) positions. For dihydro-1-ethyluracil, H atoms were abstracted from both the carbon stoms of the ethyl group. The radicals produced by H-abstraction from the C(5) or C(6) positions of the dihydropyrimidines are equivalent to those formed by H atom addition to the C(5)-C(6) double bonds of the corresponding pyrimidines. Hence the present results are helpful in

distinguishing between the spin-trapped radicals formed by H or OH addition to the C(5)-C(6) double bond of pyrimidines of γ-irradiated aqueous solutions.

7. E.S.R. study of post-radiolysis growth of spin-trapped radicals in gamma-irradiated solutions of thymine (with A. Joshi and H. Moss).

The post-irradiation growth of the spin-adduct nitroxide radical produced by the addition of the thymine-OD radical to t-nitrosobutane (tNB) in γ-irradiated deaerated D_2O solutions was investigated by e.s.r. The thymine-OD radical was formed by addition of an OD radical to the C(5) position of thymine. Growth reached a greater maximum value and was more rapid with increasing dose. At a fixed dose, growth was also greater and more rapid if oxygen was present after γ-radiolysis. Addition of a second radical to the spin-adduct nitroxide to give a diamagnetic intermediate which can regenerate the spin-adduct radical during storage in air-free and in air-saturated solutions at room temperature was inferred to be responsible for post-irradiation growth. U.V. photolysis at 260-280 nm of a solution containing the diamagnetic intermediate rapidly regenerates the spin-adduct nitroxide. The longer life-time of the diamagnetic intermediate in oxygen-free solutions may be relevant to an understanding of the anoxic sensitization by nitroxides in cellular systems.

Significance to Cancer Research and the Program of the Institute : Studies of the effects of ionizing radiation are of importance in relation to (1) radiation therapy, (2) carcinogenesis, (3) stability of the genetic pool, (4) the suppression of the immune mechanism, and (5) aging. The effects of ionizing radiation on nucleic acids are being studied in order to understand the nature of radiobiological death in normal and tumor cells. The addition of radioprotective and radiosensitizing agents is being investigated so that a therapeutic advantage may be gained. Objective 6, Approach 1.

Proposed Course of Research: To continue studies on the effects of ionizing radiation on macromolecules of biological importance. The mechanism of radioprotective and radiosensitizing agents and the interaction of radiation and cancer chemotherapy agents will be investigated.

Publications:

1. Rustgi, S., Joshi, A., Moss, H. and Riesz, P.: ESR of spin-trapped radicals in aqueous solutions of amino acids: Reactions of the hydroxyl radical. Int. J. Radiat. Biol 31: 415-440, 1977.

2. Rustgi, S., Joshi, A., Riesz, P. and Friedberg, F.: ESR of spin-trapped radicals in aqueous solutions of amino acids. Reactions of the hydrated electron. Int. J. Radiat. Biol. 32: 533-552, 1977.

3. Rustgi, S. and Riesz, P.: ESR of free radicals in aqueous solutions of substituted pyrimidines. Int. J. Radiat. Biol 33: 21-39, 1978.

4. Rustgi, S. and Riesz, P.: ESR study of spin-trapped radicals formed during the photoysis of aqueous solutions of acid amides and H_2O_2. Int. J. Radiat. Biol 33: 325-339, 1978.

5. Joshi, A., Rustgi, S., Moss, H. and Riesz, P.: ESR of spin-trapped radicals in aqueous solutions of peptides. Reactions of the hydroxyl radical. Int. J. Radiat. Biol. 33: 205-229, 1978.

6. Rustgi, S. and Riesz, P.: ESR of spin-trapped radicals in aqueous solutions of dihydropyrimidine bases. Radiation Res., in press.

7. Rustgi, S. and Riesz, P.: ESR and spin-trapping studies of the reactions of hydrated electrons with dipeptides. Int. J. Radiat. Biol, in press.

8. Rustgi, S. and Riesz, P.: Free radicals in UV irradiated aqueous solutions of substituted amides. Int. J. Radiat. Biol, in press.

9. Rustgi, S. and Riesz, P.: An ESR and spin-trapping study of the reactions of $\dot{S}O_4^-$ radical with protein and nucleic acid constituents. Int. J. Radiat. Biol., in press.

10. Joshi, A., Moss, H. and Riesz, P.: E.s.r. study of post-radiolysis growth of spin-trapped radicals in gamma-irradiated solutions of thymine. Int. J. Radiat. Biol., in press.

SUMMARY

October 1, 1977 through September 30, 1978

Summary Report

The activities of the Laboratory of Theoretical Biology (LTB) fall into several broad areas: membrane biophysics, immunology, macromolecular configurations, kinetics of metabolic systems, computational methodology, and theory related to modeling. Most of the work is theoretical, but experiments are also carried out in the laboratory. Much of the theoretical work and modeling is done in collaboration with experimental groups at NIH and elsewhere.

The work on membranes includes several areas of interest: lipid bilayer systems, vesicle-cell fusion, mechanisms of cell lysis, and diffusion of cell surface receptors in membranes (R. Blumenthal, J. Weinstein, P. Dragsten, E. Ralston). This work is both experimental and theoretical.

The studies of directed interaction between liposomes and cells are applicable to problems in cell biology and to possible uses of liposomes in clinical therapy. Fluorescence self-quenching of an encapsulated water soluble dye is used to study transfer of liposome contents to cells, and to monitor the integrity of the liposomes interacting with cells or serum. The fluorescent properties of dye-laden vesicles are also used to study specific binding of haptenated vesicles to cells. Fluorescent techniques used are flow microfluorometry, fluorometry, fluorescence microscopy and fluorescence photobleaching.

Serum components appear to inhibit competitively the saturable sites for transfer of liposome contents to cells; potential fusogenic agents (e.g. lysolecithin), local anesthetics (e.g. procaine) or changing lipids (e.g. adding cholesterol) had no effect on transfer. When vesicles containing a fluorescently labeled lipid were incubated with lymphocytes, the label did not spread out over the cell membrane. The targeting studies were extended to murine myeloma MOPC 315 cells containing endogenous surface IgA. Those cells bind liposomes having the appropriate hapten (DNP), but binding did not increase delivery to the cytoplasm. A novel approach was developed to obtain preferential delivery of liposome-encapsulated drugs to particular local areas, for example tumors and sites of infection. Liposomes were designed to release drugs primarily in areas heated a few degrees above physiological. In the presence of serum the ratio of drug release at 43° to that at 37° can be made greater than 100:1. The basic features of the approach were tested in an in vitro cell system.

In the studies of mobility of molecules in membranes it was found that surface immunoglobulins on lymphocytes have lateral diffusion coefficients, characteristic of those found for proteins in cell membranes, $2 \times 10^{-10} cm^2/sec$.

The development and application of mathematical techniques for describing receptor clustering on the plasma membrane continued (C. DeLisi). The results were incorporated into a theory of the response of sensitized basophils and mast cells which were proposed to explain, in terms of the physical chemistry of cell surface events, the biochemical pathway (histamine release, specific desensitization, non specific desensitization) selected by the cell. Aside from predicting the parameters of importance in the control of this choice, the theory also provides an explanation for the wide range of qualitative differences observed in dose response curves. The work is in collaboration with R. Siraganian, NIDR.

Work was also initiated, in collaboration with Zvi Grossman (Weizmann Institute) and Richard Asofsky (NIAID) on modeling certain aspects of systemic regulation of the immune response. The model is intended primarily for T independent antigens, but a number of regulatory features are expected to be general. Several predictions including oscillations in antibody affinity, are now being tested experimentally.

Protein secondary structures are determined by interactions between the atoms which are chemically bonded according to the protein primary sequence. Investigations (R. Jernigan, S. Szu) of the relative importance of various types of intramolecular interactions have led to approximations which permit simple calculations of probable secondary conformations. In particular, interactions between pairs of side chain atoms appear to be significantly less important than interactions between pairs of backbone atoms and between side chain atoms and backbone atoms. Electrostatic contributions to the conformational energies are the most significant class of terms. It was found that long range interactions reinforce predictions of secondary forms based on short range interactions alone. Such a redundance of interactions permits proteins to retain conformations, even in disparate environments.

The calculated relative stabilities of secondary structure regions provide a basis for postulating folding pathways. A less detailed model of macromolecular denaturation and renaturation kinetics has been applied to DNA. Conformations are specified to be simply helix or random coil. The effects of perturbation sizes and directions on overall rates were calculated in detail. Comparisons of results with experiments were more successful for denaturation than for renaturation. A similar model of protein conformation kinetics has been formulated, with helix nucleation sites determined by the local amino acid composition.

The modeling of endocrine and other metabolic and dynamic systems has continued. The lipoprotein models have been further developed to shed new light on the kinetics of cholesterol, the synthesis of plasma triglycerides, and the metabolism of low density lipoprotein triglycerides. Synthesis pathways for bile cholesterol and bile acids have been identified and quantified in T-tube patients (C. Schwartz) indicating that most of the newly synthesized cholesterol cycles through the plasma and that about 75% of bile secretions are supplied from plasma precursors. Based on these findings new experiments have been carried out to study the kinetics cholesterol and cholesterol esters on the various lipoprotein particles in plasma and their relation to bile secretion. A model was also developed for low density lipoprotein (LDL) tri-

glycerides (TG), suggesting that newly synthesized LDL is TG-rich but loses its TG in about 5-8 hours. The TG-rich particles do not exchange with extra-plasma compartments. This means that LDL particles are composed of at least 2 population of particles with distinct biological properties (Berman). New insights have also been obtained for the conversion of plasma glycerol to plasma triglycerides and the interpretation of very low density triglyceride (VLDL-TG) kinetics (Zech). The new model developed suggests that previous calculations of VLDL-TG synthesis and catabolism can be erroneous (sometimes by as much as a factor of three) if the new features claimed are neglected.

Careful analysis of low and high affinity insulin kinetics in rabbits provide considerable details of body distributions of insulin, the degree of interaction with receptors and the degrees and levels of degradation of insulins on and off receptors (M. Berman, E.A. McGuire). The analysis permits the determination of a number of receptors in vivo and the value of an effective in vivo affinity constant. Application of the developed model to similar kinetic studies in humans (by Wachslicht and Roth) is now in progress. In connection with gluconeogenesis, a detailed model for the interconversion of glucose, alanine and lactate carbons has been developed (D. Foster). The data were supplied by Dr. G. Heteney of Ottawa (dogs) and Dr. S. Hall of Ottawa (humans). This is the first detailed model developed to interrelate these substrates. An important conclusion from this analysis is the fact that the alanine (and probably lactate) carbons do not behave homogenously and that the heterogeneity must be dealt with in order to calculate correct rates of interconversion and synthesis.

Further development of SAAM modeling system has continued (M. Berman, M. Weiss), as well as its application to the study of various systems. Distribution of this program to various computer centers and its support on different computer systems has also continued.

The Laboratory of Theoretical Biology also consults with investigators on various problems and assists in the use of the SAAM program. It also supports a PDP-11/20 data processing system for which special and general programs have been developed for data processing and plotting (F. Millar).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08300-06 LTB

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

SAAM Simulation and Modeling Computer Program

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Berman, Ph.D.	Acting Chief	LTB NCI
OTHERS:	M. F. Weiss	Mathematician	LTB NCI
	R. Chabay	Staff Fellow	LTB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Theoretical Biology

SECTION

Mathematical Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.7	.7	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Continuing development of a computer system (SAAM) for the simulation, analysis and modeling of bio-kinetic systems. Further development of a conversational mode of operation, increased versatility of applications and automation are planned.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To develop a general purpose computer program for modeling biokinetic systems that may readily be used by investigators not sophisticated in mathematics or programming. Initiated in 1959, the program continues to be expanded and revised as new features are added.

Major Findings: The computational power of SAAM was further expanded. Improvements include a new extension of the Forcing Function. New commands were also incorporated into conversational SAAM making it easier to use.

Significance to Biomedical Research and the Program of the Institute: The SAAM computer program has been a most essential tool in the modeling of various systems described elsewhere in this Laboratory report. Its wide use in other centers in the United States and elsewhere is further evidence of its value in biomedical research.

Publications:

Authors: Berman, M., Weiss, M. F.: SAAM Manual. Washington, D.C., US Govt. DHEW Publication No. (NIH) 78-180, 1978, 200pp.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08301-06 LTB

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Modeling Theory and Applications: Zinc Kinetics in Man, Amino Acid Transport in Vesicles, Prostaglandin Binding and Activation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Berman, Ph.D.	Acting Chief	LTB NCI
OTHERS:	D. Foster, Ph.D.	Staff Fellow	LTB NCI
	A. Babcock, Ph.D.	Staff Fellow	LTB NCI
	R. Chabay, Ph.D.	Staff Fellow	LTB NCI
	L. Zech, M.D.	Sr. Investigator	MD NHLBI

COOPERATING UNITS (if any)
J. E. Rall, NIAMDD; T. Garvey, FDA; R. Henkin, Georgetown Univ.;, R. Aamodt, CC; I. Pastan, NCI.

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NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Analysis of various kinetic and metabolic systems by the use of mathematical models carried out in collaboration with other investigators. These include binding, internalization and activation of receptor systems (insulin, prostaglandin), zinc metabolism in man, amino acid transport and thyroglobulin synthesis. These studies also serve to test the SAAM computer program and to develop modeling theory.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: Analysis of data on metabolic systems, and further development of modeling theory and techniques through the applications of the SAAM computer program.

Major Findings: Further refinements have been introduced into the prostaglandin model (Chabay) to include the GTP effect. It was proposed that the PgE receptor is transformed into a low affinity, high efficiency state by binding to GTP. Furthermore, the affinity for GTP is increased in RS-NRK compared to NRK cells.

A model has been proposed for the transport of amino acids across vesicle membranes in the presence of Na (Babcock). The model proposes a carrier transport mechanism for amino acids with a co-transport of Na. Na increases the affinity of the carrier for amino acids. Coupled with the fact that most of the Na is transported by a separate mechanism and that the equilibrium time with vesicles for Na is slower than that for AIB - accounts for observed overshoots of amino acid uptakes by vesicles and for changes in the level of the overshoots with varying Na concentrations. The model suggests that transformed 3T3 cells membranes have an increased affinity of Na to the carrier which accounts for much of the increased transport of amino acids in the transformed cells.

Significance to Biomedical Research and the Program of the Institute: The methodology of modeling is most essential for studying the behavior of complex systems for both normal and abnormal states.

Proposed Course: This is a continuing process for the development of theory for and the application of modeling techniques.

Publications:

McGuire, R., and Berman, M.: T_4, T_3, and I^- kinetics during gestation. Endocrinology, In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08303-06 LTB

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Movement of Molecules in Membranes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R.P. Blumenthal, Ph.D.	Visiting Scientist	LTB NCI
OTHER:	J.N. Weinstein, M.D., Ph.D.	Investigator	LTB NCI
	P. Dragsten, Ph.D.	Staff Fellow	LTB NCI

COOPERATING UNITS (if any)

P. Henkart, J. Schlessinger,	IB, NCI, NIH
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C.E. Creutz, H. Pollard	CH, NIAMDD,NIH

LAB/BRANCH

Laboratory of Theoretical Biology

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Lipid bilayer membranes are used to study various aspects of membrane structure and function. Electrical properties of bimolecular lipid membranes (BLM's) are measured to study: (1) mechanisms of opening and closing ion channels; (2) properties of ionophores isolated from natural cell membranes; (3) mechanisms of immune cytotoxicity. Diffusion and patching of fluorescent surface receptors on BLM's and on cell surfaces are studied by the technique of fluorescence photobleaching recovery: a small spot on the fluorescent-labeled surface is photolytically bleached by brief exposure to an intense laser beam, and subsequent recovery of the fluorescence is monitored. Fluidity of membrane is assessed by measuring fluorescence depolarization of membranes labeled with the fluorescent probe 1,6-diphenyl-1,3,5 hexatriene (DPH). The measurements are made to study the relationship between movement of cell surface receptors and cellular activation.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To study the physical mechanisms of ion transport in reconstituted membranes. To develop the lipid bilayer membrane as an assay for ionophoric materials isolated from natural cell membranes. To study the interaction of lymphocytes with lipid bilayer membranes as a model for lymphocyte-mediated lysis of target cells. To study the role played in cell membranes by the mobility and distribution of cell surface antigens. To study the relationship between membrane fluidity and the action of cell surface receptors.

Methods Employed: The bilayer membranes are formed from natural membrane extracts, oxidized cholesterol, or pure lipids in an aperture between two electrolyte solutions. The electrical properties of the membranes are measured before and after application of an activating factor. In the fluorescence photobleaching experiments a small spot on the fluorescence-labeled cell surface is photolytically bleached by brief exposure to an intense laser beam, and subsequent recovery of fluorescence is monitored with an attenuated laser beam. In the fluidity studies membranes are labelled with DPH, and fluorescence depolarization is measured using an Aminco-Bowman spectrofluorometer equipped with Glan prism polarizers.

Major Findings: Photobleaching studies (1) An effort was made to characterize the technique of fluorescence photobleaching, and sort out potential artifacts related to beam size, depth of focus and extent of the bleaching. A modification of the apparatus was introduced to enable fast bleaches. It was established that the diffusion coefficient of a lipid probe in plasma membrane of small cells (lymphocytes) could be measured (at 10^{-7} cm^2/sec) with 10-20% reproducibility. Diffusion coefficients for lipid probes on round villous and flat smooth cells were found to be approximately equal, indicating that the presence of microvilli did not affect the measurement. (2) Mobility of surface receptor immunoglobulins on lymphocytes was measured using fluorescent labeled antibodies against the surface immunoglobulins. With IgG antibodies all the receptors were immobilized, whereas with Fab' fragments diffusion coefficients of $2x10^{-10}$ cm^2/sec were obtained. Anti theta IgG showed no immobilization at low doses. Measurements of diffusion coefficients of fluorescently labeled epidermal growth factor and α_2-macroglobulin on fibroblasts showed partial immobilization.

Reconstitution studies:

No ionophoric activity in BLM's was found for the IgE isolated form mast cells in the presence of anti-IgE. The fluorescently labeled IgE-receptor complexed was found to be associated with a BLM, when visualized in the fluorescence photobleaching apparatus.

Membrane Fluidity:

Red blood cells, which were deprived of or enriched with cholesterol showed respectively lower and higher fluorescence depolarization values using DPH.

Those values correlated well with lateral diffusion coefficients measured with lipid probes in the fluorescence photobleaching apparatus.

Theory of membrane potentials in chromaffin granules.

A model, based on the Goldman equation, has been developed to account for the relationship between measured values of the membrane potential and pH gradient in chromaffin granules. The model suggests that a negative charge is titrated on the chromaffin granule membrane, changing the permeability ratio between protons and chloride ions. The model is related to the mechanism of release of norepinephrine from chromaffin granules.

Significance for Biomedical Research and the Program of the Institute. Changes in membrane fluidity and cell surface mobility are considered to have important implications for cell transformation. This work supports Objectives 3 and 6 of the National Cancer Plan.

Proposed Course: The BLM will continue to be used as an assay for ionophores from natural membranes. Possible ionophoric activity of the purified IgE receptor from mast cells will be further investigated, since its biological activity has been associated with Ca^{2+} influx. The photobleaching technique will be applied both to surface receptors incorporated into planar bilayers and to mobility of cell surface receptors for hormones, immunoglobulins, and transplantation antigens. We also hope to measure membrane flow using this technique. The fluorescence depolarization studies will be extended to measuring membrane preparations of mice fed with diets deficient in essential fatty acids, and relating the measurements to function of surface receptors. We plan to measure potentials in single cells using fluorescent probes.

Publications:

Blumenthal, R., Weinstein, J.N. and Henkart, P.: Lipid model membrane studies of immune cytotoxic mechanisms. In Miller, M.W. and Shamoo, E.A. (Eds.): Adv. in Experimental Medicine and Biology. New York, Plenum, 1977, pp. 495-509

Blumenthal, R. and Shamoo, A.E.: Incorporation of transport molecules into black lipid membranes. In O'Brien, R.D. (Ed.): The Receptors. A Comprehensive Treatise. New York, Plenum, 1978, in press.

Wolf, D.E., Schlessinger, J., Elson, E.L., Webb, W.W., Blumenthal, R. and Henkart, P.: Diffusion and patching of macromolecules on planar lipid bilayer membrane. Biochemistry 16: 3476-3483, 1977.

Kafka, M.S., Blumenthal, R, Walker, G.A. and Pollard, H.B. The effect of dopamine-B-hydrocylase on the electrical conductance of bimolecular lipid membranes. Membrane Biochemistry 1978, In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08306-06 LTB

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Kinetic Modeling of Human Plasma Lipoprotein Metabolism

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Berman, Ph.D.	Acting Chief	LTB	NCI
OTHERS:	L. Zech, M.D.	Research Associate	MDB	NHLBI
	D. Foster, Ph.D.	Staff Fellow	LTB	NCI
	C. Schwartz, M.D.	Veterans Administration, Richmond, VA		
	L. Swell, Ph.D.	Veterans Administration, Richmond, VA		

COOPERATING UNITS (if any) Dr. C. Malmendier, Univ. of Brussels, Belgium; Molecular Disease Branch, NHLBI, NIH; Drs. S. Grundy, Veterans Admin, San Diego, CA; Dr. W. Fisher, Univ. of Fl, Gainesville, FL; Dr. W. Hazzard, Univ. of Washington, Seattle, WA; Dr. Steinberg, UCSD, School of Medicine, San Diego, CA

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Laboratory of Theoretical Biology

SECTION
Mathematical Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Kinetic models of plasma apoproteins, cholesterol and triglyceride are being constructed based on data from experiments in man. The models are used to integrate plasma lipoprotein interactions with enzymes and receptors and to provide a better understanding of plasma lipoprotein synthesis and metabolism in health and disease. The models are particularly useful for the rigorous testing of hypothesis, the design of experiments, and the quantification of the effects of various perturbations.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To develop a qualitative and quantitative understanding of lipoproteins metabolism in man and to identify abnormalities and drug effects through the modeling of apoprotein, cholesterol, and triglyceride kinetics. Because of the need for diverse extensive data on a variety of patients, collaboration with several experimental groups is maintained.

Methods Employed: Mathematical modeling is the tool used for the integration and analysis of the data. This is performed with the help of the SAAM computer modeling program.

Major Findings: Modeling of Low Density Lipoprotein triglycerides (LDL-TG) kinetic in humans (Berman, Malmendier) suggests that LDL is a heterogeneous population with respect to its TG contents. About 10% of the LDL particles are 'triglyceride rich' and do not have the ability to exchange with an extra-plasma compartment as most LDL particles do. These particles are the newly produced LDL particles and they lose most of their triglycerides in the first 6 hours to become 'regular' LDL particles. The remaining 5-10% of the original TG on the particles seem to follow the kinetics of the apo-B apoproteins, at least over the first 48 hours.

An initial detailed model for the synthesis of cholesterol from mevolovic acid precursors and the contribution of exogenous and endogenous cholesterol to the formation of bile acids and bile cholesterol - has been developed. The model shows that in T-tube patients about 70% of the cholesterol used for bile secretion comes from plasma and only 30% from newly synthesized cholesterol. Details as to which of the plasma lipoproteins contribute to this are now being investigated (Schwartz, Swell).

Significance to Biomedical Research and the Program of the Institute: Modeling is important to identify the abnormalities in metabolism responsible in hyperlipemics, diabetics, and other metabolic disorders, given a very complicated system with multiple interactions. It is hoped that by identifying the mechanisms responsible for abnormalities in lipid metabolism and their relations with intermediate metabolism, appropriate treatment can be more specifically geared to abnormalities. Clinically this is relavant to atherosclerosis and disorders associated with athersclorsis such as cardiovascular desease, diabetes, cachexia, and other metabolic disorders involving lipids.

Publication:

Berman, M., Hall, M., III, Levy, R., Eisenberg, S., Bilheimer, D.W., Phair, R.D., and Goebel, R.H.: Metabolism of apo-B and apo-C lipoprotein in man: Kinetic studies in normals and hyperlipoproteinemics. J. Lipid Res. 19: 38-56, 1978.

Blum, C.G., Levy, R.I., Eisenberg, S., Hall, M., III, Goebel, R.H., and Berman, M.: High density lipoprotein in man. J. Clin. Invest. 60: 795-807, 1977.

Schwartz, C.C., Berman, M., Vlahcevic, Z.R., Halloran, L.G., Gregory, D.H., and Swell, L.: Multicompartmental analysis of cholesterol metabolism in man. I. Characterization of the hepatic bile acid and biliary cholesterol precursor sites. J. Clin. Invest. 61: 408-423, 1978.

Malmendier, C., and Berman, M.: Endogenously labeled low density lipoprotein triglyceride and apoprotein B kinetics. J. Lipid Res. In press.

Berman, M.: Kinetic Analysis of Lipoprotein Turnover Data. In Eisenberg, S. (Ed.): Progress in Biochemical Pharmacology: Lipoprotein Metabolism. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 CB 08312-05 LTB

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Computer Applications: Programming and Data Processing

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: F.K. Millar, Ph.D. Computer Specialist LTB NCI

COOPERATING UNITS (if any)

None

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NCI, NIH, Bethesda, Maryland 20014

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0.6	0.6	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To develop software for data processing and other computer applications in the investigation of selected biomathematical problems.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To facilitate the investigation of biomedical research problems through the application of computer programming and computer data processing.

Methods Employed: Use is made of the PDP-11/20 computer of this laboratory, the National Bureau of Standards Univac 1108 computer, and the central NIH computer facility. The projects reported may be collaborative efforts with investigators in other units, as shown below.

Major Findings:

Computer Applications:

(1) Processing and plotting of data from liquid spectrometers; comparison of isoaccepting species of tRNA isolated from normal and malignant cells (Dr. J. F. Mushinski, C/LCBGY).

Data collected on punched paper tape are reduced and analyzed on the PDP-11/20. Final results are plotted directly on the on-line CalComp plotter and are also stored on magnetic tape for future retrieval, e.g., for additional analysis on the NIH central computers.

(2) The general purpose plotting package, for the CalComp plotter on-line to the PDP-11/20, and the associated edit program are now essentially completed and in use. Together the two programs provide procedures whereby the user formulates, tests and re-designs graphs as desired. Under default procedures, a graph of the observed and calculated data generated as output from a run of the SAAM program (Project No. ZO1 CB 08300-06 LTB) will result.

(3) A project to augment the PDP-11/20 system by introduction into the configuration of a 1200-baud terminal as back-up printer is near completion. This change has involved modifications to the system monitor as well as additional hardware. Advice and assistance have been provided by members of the Computer Systems Laboratory, DCRT.

Significance to Biomedical Research and the Program of the Institute: Computer programming applications contribute to the investigation of research problems by providing (1) accurate and efficient methods for handling laborious calculations of data and visualizing results, (2) the ability to store and retrieve large amounts of data, and (3) opportunities for testing mathematical models based on experimental results.

Proposed Course: Programs will continue to be developed and computational techniques to be applied in response to computer-related needs of the scientific staff.

Publications:

Haeberli, A., Millar, F.K., and Wollman, S.H.: Accumulation and localization of radiocalcium in the rat thyroid gland. Endocrinol. 102: 1511-1519, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08317-04 LTB

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Conformational Transitions in Polypeptides and DNA

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: R. L. Jernigan, Ph.D. Theoretical Physical Chemist LTB NCI

OTHER: S. C. Szu, Ph.D. Visiting Associate LTB NCI

COOPERATING UNITS (if any)

None

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Laboratory of Theoretical Biology

SECTION

Mathematical Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose is to elicit molecular details of conformational kinetics in polypeptides and DNA. An eventual aim is to understand the mechanisms and pathways of DNA double helix formation, of protein folding, and of denaturation and renaturation. A mathematical model of the rates for passing between the various configurational states was previously formulated. Molecular states are determined by specification for each residue of a helical or random coil conformation. A comprehensive series of experiments on viral DNA indicated that the denaturation rates depend strictly upon the instantaneous conformational state; whereas, the constant rates of renaturation depended only upon initial and final conditions. The simplest theoretical model yields qualitative agreement with the denaturation experiments and the series of renaturation experiments from the same initial condition. Partial agreement with the group of renaturation experiments with the same final condition is achieved only by including a bimolecular helix initiation step with properly matched pairs of strands. Comparison of all experimental and calculated rates yields $5 \times 10^{4} min^{-1}$ as the approximate step rate for melting a single base pair.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To determine the molecular details of conformational kinetics in macromolecules. An eventual aim is to understand the mechanisms and pathways of protein and DNA denaturation and renaturation kinetics.

Methods Employed: A mathematical model of the rate for passing between the various configurational states was previously formulated. Molecular states are determined by specification for each residue or base pair of a helix or random coil conformation. Averages are performed over all possible conformations.

Major Findings: The previous model, appropriate for polypeptides, has been extended to treat conformational relaxation in deoxyribonucleic acids. A major reason for this extension is the possibility of making direct comparisons with the comprehensive series of experiments by Record and Zimm. Effects of perturbation direction and size were calculated; strong dependences were observed, mostly in agreement with corresponding experiments. The effects of including a bimolecular reaction for the formation of the first DNA helix unit and proper alignment of the pair of strands were investigated.

Significance to Biomedical Research and the Program of the Institute: A basic understanding of denaturation and renaturation of DNA and of proteins is essential for understanding cellular processes on a molecular basis. For example, such comprehension is necessary before detailed effects of external chemical agents can be determined.

Proposed Course: No single model above has been adequate to achieve agreement with all experiments. We will attempt to construct simple models consistent with all data by utilizing the modeling computer program SAAM. Such fundamental models could be useful to suggest the form of more detailed molecular models.

Publications:

Szu, S.C., and Jernigan, R.L.: DNA conformational kinetics. Biopolymers. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 CB 08320-03 LTB

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Macromolecular Conformations

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. L. Jernigan, Ph.D.	Theoretical Physical Chemist	LTB NCI
OTHER:	S. C. Szu, Ph.D.	Visiting Associate	LTB NCI

COOPERATING UNITS (if any) T. T. Wu, M.D., Ph.D. Northwestern University;
H. Bilofsky, Ph.D. Bolt, Beranek & Newman, Inc. Camb., MA;
E. Kabat, Ph.D. NCI, NIH; Columbia Univ., Presbyterian Hospital N.Y.;
S. Bourgeois, Ph.D. The Salk Institute for Biological Studies, CA

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INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.7	1.7	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A new method has been developed to choose probable protein secondary conformations. Direct interatomic interaction energies are calculated to yield probabilities of various conformers. The side chain atoms are taken at fixed positions relative to the polypeptide backbone. Charges are assigned, for ionized groups, from pK values, and, for atoms in polar bonds, from small molecule dipole moments. Approximate energies of all possible helix and β-strand segments are calculated. Several methods of selecting conformations have been investigated. In the most successful method, non-overlapping secondary regions are chosen sequentially; those with the most favorable energies are selected first.

Project Description:

Objectives: To develop adequate methods to determine the most significant molecular conformations. In particular, we wish to predict protein conformations from the sequences and to determine the relative importances of the various intramolecular interactions.

Methods Employed: A new method has been developed to calculate relative probabilities of secondary structure regions at all positions in a protein. Side chain atoms are taken at fixed positions, as determined by their average positions from X-ray crystal studies. Approximations of the interatomic interaction energies yield probabilities of secondary conformations. Several methods were devised to choose from large sets of possible regular secondary structures.

Major Findings: Side chain-backbone electrostatic interactions are relatively insensitive to the approximation of fixed side chain positions. Calculated side chain - side chain interactions are more sensitive but are reduced by side chain flexibility, tertiary interactions and the intervention of solvent molecules. Three methods of selecting the most favorable set of secondary conformations were compared: 1) choosing the set of conformations with the lowest total energy, 2) calculating, for each residue, relative α-helix and β-strand probabilities by summing over probabilities of all secondary regions spanning a given residue, and 3) selecting the lowest energy form and picking successive regions of low energy which do not overlap previously chosen regions. Best results are achieved with the last method. Predictions are 60 to 90% correct, in comparisons with known crystal structures. Best predictions are achieved for helix and in molecules with relatively long regular helix and β-strands.

Significance to Biomedical Research and the Program of the Institute: Development of a reliable method for predicting protein conformations from their sequences would permit a better understanding of protein structure and function, as well as the effects of mutants and species variations.

Proposed Course: Further evaluation and refinement of the methods and associated parameters will be pursued. These methods will be applied to other molecules. Previously, only regions of regular repeating conformations were considered; the methods will be extended to include less regular conformations.

Publications:

Wu, T.T., Szu, S.C., Jernigan, R.L., Bilofsky, H., and Kabat, E.A.: Prediction of β-sheets in immunoglobulin chains. Comparison of various methods and an expanded 20 x 20 table for evaluation of the effects of nearest neighbors on conformations of middle amino acids in proteins. Biopolymers. 17: 555-572, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08322-03 LTB

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Quantitation of the Requirements for Ligand Induced Basophil Degranulation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: C. DeLisi, Ph.D. Senior Investigator LTB NCI

COOPERATING UNITS (if any)
R. Siraganian, M.D., Ph.D. LMI,NIDR,NIH

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NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A method was developed for relating the amount of histamine released at low doses of antigen to the number of crosslinked receptors on the basophil plasma membrane. The method was applied to AgE, con A and a IgE mediated histamine release from human basophils.

PHS-6040
(Rev. 10-76)

Project Descriptions:

Objectives: To develop an understanding of the relationship between the thermodynamics and kinetics of ligand cell-bound receptor interactions, and the requirements for lymphocyte activation.

Methods Employed: Mathematical Modeling

Major Findings: A method was developed for determining from the analysis of the low dose response, the number of allergen molecules required to induce release of histamine from basophil compartments. The method was applied to Antigen E, con A and anti IgE induced release from human basophils in the presence and absence of heavy water. The results indicate that a threshold in the number of crosslinked allergens must be met before any histamine can be released. The average numbers were four per compartment for con A and AgE, and ten per compartment for anti IgE. The analysis also suggests that there are fewer than ten compartments per cell.

Histamine release in the presence of heavy water begins at an allergen concentration which is an order of magnitude smaller than in ordinary buffer. Transformation and analysis of the data, however, indicates no difference in the number of active antigens required to induce release, suggesting that heavy water affects the efficiency of signal transduction.

Proposed Course: The magnitude of the threshold is predicted to be a function of antigen valence. Future experiments will involve penicylloyl derivatives of various valences in order to study the valence dependence of the threshold for activation. More generally, methods will be developed for analyzing low dose data in order to sort out the parameters which determine the minimal requirements for histamine release.

Significance to Biomedical Research and the Program of the Institute: The problem of determining and quantitating the parameters which effect the initial stages of signal transduction in basophils, is an important aspect of the attempt to understand the nature of the immediate allergic reaction. More generally, however, the basophil system may serve as a model from which to build a deeper understanding of the relation between the binding of ligand to cell surface receptors and subsequent biochemical events and factors which regulate them.

Publications:

DeLisi, C.: Some mathematical problems in the initiation and regulation of the immune response. Math. Biosci. 35: 1-26, 1977.

DeLisi, C.: Physical chemical aspects of cellular selection in an immune response. In Bell, G.I. (Ed.): Theoretical Immunology. New York, Marcel Dekker, 1977, pp. 215-242

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 CB 08323-03 LTB

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Quantitation of Immunochemical Assays

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: C. DeLisi, Ph.D. Senior Investigator LTB NCI

COOPERATING UNITS (if any)
G. Doria, Univ. of Rome

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Laboratory of Theoretical Biology

SECTION
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INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Analysis of the hemolytic plaque assay continued, with particular emphasis on the experimental conditions which must be fulfilled for inhibition experiments to yield thermodynamic information. Work on a related problem was also initiated, viz, obtaining from equilibrium binding data, the distribution of equilibrium constants characterizing the interaction of a heterogeneous receptor population with homogeneous ligand. The problem was approached by a method of moments.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To quantitate certain immunological assays and delineate conditions under which physical chemical information can be obtained.

Methods Employed: Mathematical Modeling

Major Findings: Application of plaque inhibition theory led to the reconciliation of a wide body of apparently conflicting results on IgM affinity maturation. Specifically I found that all workers who reported absence of maturation were unknowingly using assay conditions which the theory predicts are insensitive to affinity variations, while those who reported maturation were using conditions which theory predicts are optimal or near optimal.

For the receptor heterogeneity problem, Monte Carlo simulations indicate that for a wide range of distributions, the method should yield the first two moments to within experimental error, and for distributions which do not deviate too severely from a gaussian, the method should yield the entire distribution.

Significance to Biomedical Research and the Program of the Institute: In order to develop a complete understanding of the relationship between the immune system and tumors, physical chemical aspects of their interaction must be characterized. This project is working toward that goal.

Proposed Course: The hemolytic plaque theory is currently being used, and will continue to be used in the coming year, to design experiments which will allow new information on antibody secretion rate distributions to be obtained. The work will be in collaboration with Dr. Gino Doria (Rome).

Publications:

DeLisi, C.: Detection and analysis of recognition and selection in the immune response. Bull. Math. Biol. 39: 705-719, 1977.

Thakur, A., and DeLisi, C.: Theory of ligand binding to heterogeneous receptor populations characterization of the affinity distribution function. Biopolymers. 17: 1075-1089, 1978.

DeLisi, C.: Characterization of receptor affinity heterogeneity by scatchard plots. Biopolymers. 17: 1385-1386, 1978.

DeLisi, C., and Thakur, A.K.: An analysis of the limits of resolution of binding experiments as assays for affinity heterogeneity. Immunochem. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 CB 08330-02 LTB

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Mathematical Models of Carcinogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: C. DeLisi, Ph.D. Senior Investigator LTB NCI

COOPERATING UNITS (if any)
L. Liotta, M.D., Ph.D. LP, NCI, NIH

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NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The work in this area attempts to formulate a broad conceptual framework within which one can incorporate as much of the phenomonology of carcinogenesis as possible, and to develop mathematical models for specific aspects of carcinogenesis within this framework, that lead to testable predictions or suggestions for original experiments. Efforts are currently directed toward both fundamental and applied theory. The former category presently includes models of the host defense interacting with the primary tumor, and models of metastasis formation. In the latter category a specific model for breast cancer epidemiology has been proposed.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To develop mathematical models which describe and lead to a fuller understanding of factors influencing the age incidence of female breast cancer, the dynamics of metastasis formation and aspects of the interaction of the immune system with tumor associated antigens.

Methods Employed: Mathematical Modeling.

Major Finding: The breast cancer model fits a wide range of epidemiological data and accounts at once for the several age incidence patterns observed world wide; viz, a continuous increase in incidence with age; an increase with a single interruption at menopause and an increase which continues until the menopause and then declines. One clear prediction of the theory is that in Japan the effective at risk population is only about two percent of the total population, whereas in Western countries the figure is about an order of magnitude larger. Carefully planned genetic studies will be required to test this. The model is also able to simulate the effect of various risk factors on incidence and here too, leads to new predictions.

The work on metastasis has been part of a collaborative effort with Dr. Lance Liotta, Lab. of Pathology, DCBD. We have shown that all available experiments which report the dynamics of artificial metastases, with and without treatment of the host, can be fitted by a two compartment model, the first compartment representing cells which have lodged in the vessels of the target organ, and the second, cells which have penetrated the tissue.

The model of tumor cell - host defense interaction indicates that even with a simple two variable non linear system, much of the apparent irregularities in the literature may be simulated. It may thus be crucial to consider biological variability within the context of the dynamical structure of the system, in trying to understand the host response to tumor associated antigen.

Proposed Course: Discontinued for lack of support.

Significance to Biomedical Research and the Program of the Institute: Understanding the factors which influence the growth and spread of malignancies is a central problem in cancer research and the goal of our effort is to develop such an understanding by using mathematical modeling to analyze experimental data.

Publications:

Rescigno, A., and DeLisi, C.: Immune surveillance and neoplasia. II. Bull. Math. Biol. 39: 487-497, 1977.

Liotta, L.A., DeLisi, C., Saidel, G., and Kleinerman, J.: Micrometastasis formation. A probabalistic model. Cancer Letters. 3: 203-209, 1977.

Liotta, L.A., Saidel, G., Kleinerman, J., DeLisi, C.: Micrometastasis Therapy Theoretical Concepts. In Day, S.B. (Ed.): Proc. Cancer Metastasis Workshop. New York, Raven Press, 1977, pp. 485-492

Liotta, L.A., DeLisi, C., Vembu, D., and Boone, C.: Micrometastases: model systems. In Weiss, L. (Ed.): Pulmonary Metastases. In press.

Liotta, L.A., and DeLisi, C.: Mathematical method for quantitating tumor cell removal and tumor cell invasive capacity in experimental metastases Cancer Res. 37: 4003-4014, 1977.

DeLisi, C.: The age incidence of female breast cancer: simple models and analysis of epidemiological patterns. Math. Biosci. 37: 245-266, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08331-02 LTB

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Glucose-Insulin Kinetics Modeling of Carbohydrate Metabolism

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Berman, Ph.D.	Acting Chief	LTB NCI
	D. Foster, Ph.D.	Staff Fellow	LTB NCI
OTHERS:	E. A. McGuire, Ph.D.	Staff Fellow	EFS NIAMDD
	R. Andres, M.D.	CPB, GRC, NIA, Baltimore, MD	
	J. Tobin, M.D.	CPB, GRC, NIA, Baltimore, MD	
	J. Roth, M.D.	Chief	DB NIAMDD
	G. Hetenyi, M.D., Ph.D.	Univ. of Ottawa	
	S. E.H. Hall, Ph.D.	Ottawa Civic Hospital	

COOPERATING UNITS (if any)
Clinical Physiology Branch, GRC, NIA, Baltimore, Diabetes Branch, NIAMDD, NIH, Bethesda, Dept. of Physiology, Univ. of Ottawa, Div. of Metabolism, Ottawa Civic Hospital, Ontario

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Laboratory of Theoretical Biology

SECTION
Mathematical Biology

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1.5	1.6	0

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☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Kinetic models of the glucose regulatory systems are being developed for man and other animals in collaboration with several experimental groups. These include models of the distribution, metabolism, and receptor binding of insulin using data both radioiodinated and native insulin models of the production and utilization of glucose as elucidated by tracer studies with labeled glucose alanine and lactate, and models of the role of insulin in the control of glucose utilization and production. The models are being used to gain insight into the metabolic changes which take place in various conditions of altered glucose homeostasis such as obesity and diabetes.

Kinetic models of the glucose regulatory system, including the insulin subsystem, are being developed for man and other animals from collaborative studies with experimental groups and from data in the literature.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To develop a qualitative and quantitative understanding of glucose homeostasis including the role which various hormones insulin plays in the production and utilization of glucose and the role which glucose plays in the secretion of insulin in normal and perturbed metabolic states.

Major Findings: Detailed analysis of the kinetics of high (pork) and low (guinea-pig) affinity insulin in the rabbit indicate that in addition to a general total body distribution space for both insulins, there was a specific receptor domain for the high affinity insulin. By measuring TCA precipitable and soluble and antibody-bindable plasma activities, the following additional details emerged: 1) There are two states for insulin receptors - one equilibrating with plasma in about a minute, and the other in about 5-20 minutes. The second state may be an aggregation state for insulin. 2) There is a partial degradation of insulin from the first state, yielding a TCA precipitable fragment which is no longer specific for the receptor. 3) There is irreversible loss (or degradation) of insulin from the second receptor state suggesting an internalization path. 4) There is some partial and irreversible degradation of insulin occuring from 'deep' total body distribution compartments. 5) There is inhomogeneity (~10%) in both the high and low affinity insulins which may be due to 2 labeling sites on the insulin molecule. Quantification of the magnitude of the pathways was possible. Experiments to validate these further and the extension of the studies to human subjects is in progress.

A greater refinement in the model describing the interconversion of carbon atoms between alanine, lactate and glucose has been achieved from new data supplied by Dr. Hetenyi in normal and diabetic dogs (Foster). The new analysis confirms the previous suggestion that alanine carbons are not handled uniformly. The same also seems to hold for the lactate carbons. The model was applied to show differences in metabolic pathways between normal and pancreatectomized dogs. A similar model has also been proposed for the study of carbon interconversions in normal and diabetic humans in collaboration with Dr. Susan Hall.

Significance to Biomedical Research and the Program of the Institute: Carefully tested models provide predictions as to how a complicated regulatory system will respond to a given perturbation. The clinically or experimentally observed responses seen in various altered metabolic states can thus be analzed using such models to gain insight into the basic perturbations which have accurred in the states. Obesity, diabetes, aging and the cachexia of advanced cancer are examples of metabolic conditions in which glucose homeostasis is perturbed and which can thus be better understood by use of these models.

Proposed Course: 1) To study the regulatory behavior of the glucose-insulin system in an attempt to identify the primary perturbations in the obese, diabetic and aged subjects for which data are available. 2) To extend the insulin model to account for differences between studies using radioiodinated and native insulin, and to include new information on the binding and degradation of insulin. 3) To study the biological effects of insulin action.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08332-01 LTB

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Models of Regulation of Antibody Secreting Cell Production

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C. DeLisi, Ph.D.	Senior Investigator	LTB NCI
OTHER:	Zvi Grossman, Ph.D.	Visiting Associate	LTB NCI

COOPERATING UNITS (if any)

R. Asofsky, M.D., LMI, NIAID, NIH

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Laboratory of Theoretical Biology

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Mathematical Biology Section

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Mathematical models focusing on cellular control by surface events have been extended to include systemic control processes.

PHS-6040
(Rev. 10-76)

Project Descriptions:

Major Findings: The observations of interest are the sustained synchronous cycles in the number of antibody secreting cells, and the discontinuous, staircase like increase in cell number seen during the first few days of the response. The main features of the model on the basis of which these results are interpreted are time delays in the reseeding of the local B cell pool, and inhibition of stimulation by antibody feedback.

In addition to describing various characteristics of cyclical antibody production, the model makes a variety of predictions related to some of its principal hypotheses, including the effects of the timing and magnitude of passive antibody administration. The model also predicts, in agreement with observation, the existence of an optimal antigenic dose, high and low zone unresponsiveness, and high zone tolerance. In addition, it predicts a totally new result: cyclical variations in antibody affinity.

An analysis of steady state properties indicates that for a wide range of parameters the system possesses multiple steady states and limit cycle behavior. The influence of antigen concentration on the frequency of oscillation is found to be weak, in agreement with observation, but its influence on amplitude is strong. The steady state actually attained depends upon the relation between the initial antigen concentration and certain thresholds, as well as the presence or absence of passive antibody.

Proposed Course: Experiments are planned to test predictions of affinity oscillations and feedback control by passive antibody. The model in its present form focuses on antigens which are T independent. It will be extended to include suppressor T cells.

Significance to Biomedical Research and the Program of the Institute: Understanding the basis of immune recognition and control (including paralysis and unresponsiveness) would clearly be an important step in understanding conditions under which a host's immune system might be effective in eliminating or holding in check incipient tumors, and in providing a rational basis for immunotherapy. This project is a link between assay quantitation and immune surveillance models.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08333-01 LTB

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Models for the Control of Degranulation and Desensitization of Basophils

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C. DeLisi, Ph.D.	Senior Investigator	LTB NCI
OTHER:	M. Buas, Ph.D.	Staff Fellow	LTB NCI

COOPERATING UNITS (if any)

R. Siraganian, M.D., Ph.D. LMI,NIDR,NIH

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1.7	1.5	0.2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A kinetic theory of cell surface receptor clustering, and its relation to the kinetics of histamine release was developed for divalent ligands (allergens) interacting with divalent receptors (IgE). The model allows for specific desensitization as well as release, and incorporates the threshold requirement for release which was suggested by a previous analysis.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: An effort is being made to understand the relationship between the physical chemistry of cell surface events, and the degranulation or desensitization of basophils. A relatively simple mathematical model has been developed which integrates the physical chemical parameters which are important determinants of the size and distribution of cell surface clusters, and incorporates a simple threshold hypothesis which relates the extent of crosslinking to release and desensitization. We have shown that the wide variability in dose response curves (biphasic unimodal curves; bimodal curves; curves which rise to a plateau; curve which peak and then drop to a non zero plateau) can be consequences of differences in certain physical chemical parameters, and have identified these parameters. Work in the immediate future will involve direct experimental tests by looking for correlations between parameter changes and the predicted changes in dose response curves. Identifying the relation between cell surface events (which are controlled by certain physical chemical parameters) and the biochemical pathway which a cell follows (response, non response, desensitization) is a fundamental problem in cell biology which may have wide ranging implications bearing on cellular control mechanisms.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08334-06 LTB

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Mechanisms of Liposome-cell Interaction

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R.P. Blumenthal, Ph.D.	Visiting Scientist	LTB NCI
OTHER:	J.N. Weinstein, M.D., Ph.D.	Investigator	LTB NCI
	P. Dragsten, Ph.D.	Staff Fellow	LTB NCI
	E. Ralston, Ph.D.	Visiting Fellow	LTB NCI

COOPERATING UNITS (if any)

P. Henkart, Ph.D., S.O. Sharrow, J. Wunderlich, M.D. IB, NCI, NIH

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NCI, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Lipid bilayer vesicles containing a high concentration of the fluorescent dye carboxyfluorescein are used to study mechanisms of interaction between liposomes and human peripheral blood lymphocytes. Self-quenching largely prevents the dye from fluorescing as long as it remains trapped at high concentration in vesicles. Relief of self-quenching as the dye is released into the much larger volume of the cell is a measure of transfer of liposome contents to cell. Transfer into the cell is distinguished from stable adsorption onto the cell surface by fluorescence microscopy, fluorometry and flow microfluorometry. Vesicles made up of neutral lipids, which are in their liquid-crystalline state, transfer their contents to cells, with no significant amount of stable adsorption to the cell surface. The effects of lipid composition, potential "fusagens" and serum components which inhibit transfer are studied. When vesicles containing a fluorescently labeled lipid are incubated with lymphocytes, the label does not spread out over the cell membrane.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To study the physical mechanisms of interaction of liposomes with cells as a probe for membrane structure and function and as a model for membrane-membrane interaction in biological systems. To develop fluorescent techniques for studying vesicle cell interactions, with the possibility of designing liposomes as vehicles for delivering drugs and other materials to cells.

Methods Employed: Single-bilayer vesicles containing different amounts of carboxyfluorescein are formed by sonication from dioleoyl phosphatidyl choline (DOL) or other phospholipids. After filtration through a millipore filter free dye is removed on a Sephadex-G50 column. At high concentrations (100mM-200mM) fluorescence of carboxyfluorescein is self-quenched. Relief of self-quenching when the dye is diluted from vesicles into the medium is a measure of leakage of dye from vesicles. The vesicles are incubated with human peripheral blood lymphocytes, and the resulting fluorescence is observed by microscopy, by fluorometry, and by use of the fluorescence-activated cell sorter. In the studies of the fate of a fluorescent lipid label, vesicles are made up of DOL + 5% (mol/mol) N-4-nitrobenzo-2-oxa-1,3-diazole phosphatidyl ethanol amine.

Major Findings: (1) There was no lag period within one minute in the rate of transfer of carboxyfluorescein from liposome to cell. This indicates that the binding step to the saturable sites (see Z01 CB 08303-06) is a relatively fast process. (2) Crystalline bovine serum albumin is a competitive inhibitor of the transfer through the saturable sites. High-density (HDL) and low-density (LDL) lipoprotein inhibit transfer with a mechanism consistent with competitive inhibition. Their inhibitory effect was not as potent as that of bovine serum albumin at concentrations found for those components in 10% fetal calf serum. (3) Bovine serum albumin had no significant effect on leakage of carboxyfluorescein from liposomes; HDL and LDL did affect the leakage at low vesicle concentration. (4) Possible fusogenic agents such as ethanol, methanol, decane, DMSO, the Ca^{2+} ionophore A23187, valinomycin, octane and concanavalin A all had no effect on transfer. (5) The local anaesthetic procaine had no effect. (6) Vesicles made up of egg yolk lecithin or DOL + 50% (mol/mol) cholesterol transferred carboxyfluorescein to lymphocytes to the same extent as control (DOL) vesicles. (7) DOL liposomes obtained from the small unilamellar vesicle peak of a sepharose 4B column exhibited approximately the same rate of transfer as control vesicles obtained from a sephadex column; multilamellar vesicles coming from the void volume of the sepharose 4B column transferred their contents at a slower rate. (8) Vesicles made up of DOL and lysolecithin up to concentrations of 20% (mol/mol) exhibited the same rate of transfer as control (DOL) vesicles; the DOL-lysolecithin vesicles were more leaky and on the average smaller, but correcting for those effects did not contribute to a significant difference in liposome-mediated transfer. (9) Vesicles made up of DOL and 5% (mol/mol) of the fluorescent lipid probe N-4-nitrobenzo-2-oxa-1,3 diazole phosphatidyl ethanol amine showed similar kinetics of increase in fluorescence when incubated with lymphocytes as carboxyfluorescein-containing DOL vesicles; fluorescence microscopy, however,

indicated that the lipid label did not spread over the plasma membrane. (10) Studies of the time course of passage of dye into cells showed that liposome-mediated entry followed the same kinetic pattern as free carboxyfluorescein, but the influx was 10-fold faster. Efflux of dye from cells was the same whether the dye had entered from the medium or via liposomes. In the absence of metabolic inhibitors, steady state was reached at a concentration ratio of about 40 between free external and internal carboxyfluorescein. The data suggest transport of carboxyfluorescein by an active exit pump, which is blocked by metabolic inhibitors, and is similar to the exit pump for folate and antifolate compounds in lymphocytes.

Significance for Biomedical Research and the Program of the Institute. The vesicle-lymphocyte experiments have a direct bearing upon attempts to develop drug-loaded vesicles targeted for specific tumor cells. This work supports Objectives 3 and 6 of the National Cancer Plan.

Publications:

Blumenthal, R., Weinstein, J.N., Sharrow, S.O., and Henkart, P.: Liposome-lymphocyte interaction: Saturable sites for transfer and intracellular release of liposome contents. Proc. Natl. Acad. Sci. USA 74: 5603-5607, 1977.

Weinstein, J.N., Blumenthal, R., Sharrow, S.O., and Henkart, P.A.: Antibody-mediated targeting of liposomes. Biochim. Biophys. Acta, In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08335-02 LTB

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

"Targeting" Liposomes for Selective Interaction with Particular Cells and Tissues

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.N. Weinstein, M.D., Ph.D.	Investigator	LTB NCI
OTHER:	R.P. Blumenthal, Ph.D.	Visiting Scientist	LTB NCI
	P. Dragsten, Ph.D.	Staff Fellow	LTB NCI
	E. Ralston, Ph.D.	Visiting Fellow	LTB NCI

COOPERATING UNITS (if any) P. Henkart, S.O. Sharrow, J. Wunderlich, L. Leserman, IB, NCI; M. Yatvin, and W. Dennis, Univ. of Wisconsin; R. Kahn, J.S. Flier, DB, NIAMDD; J. Goerke and R. Hamilton, CVRI, Univ. Calif. S.F.

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NCI, NIH, Bethesda, Maryland 20014

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2	2	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In the past year we have explored ways of directing liposomes to particular target cells or tissues. When hapten-modified human lymphocytes were incubated with hapten-modified liposomes and the corresponding bivalent antibody, liposomes bound in large numbers to the cell surface. However, contents of the bound vesicles did not enter the cells. When murine myeloma cells (MOPC 315, secreting an IgA anti-trinitrophenyl) were incubated with liposomes carrying a nitrophenyl hapten, again there was binding but no delivery of liposome contents into the cells. We are currently extending these studies to phagocytic cell lines.

We have designed liposomes to release an entrapped drug preferentially at temperatures obtainable by mild local hyperthermia, for example in the treatment of tumors. This system makes use of increased release of liposome contents near their phase transition temperature. In the presence of serum the ratio of drug release at 43° to that at 37° can be made greater than 100:1. We have tested the approach in an in vitro cell system and are preparing now for in vivo studies.

Project Description:

Objectives: To investigate mechanisms of interaction between liposomes and cells, as applicable both to problems in cell biology and to the possible uses of liposomes in clinical therapy. Within this broad context,

(1) To explore the use of antigen-antibody interactions to achieve selective association of liposomes with particular cell types;

(2) To develop synergistic interactions between liposomal drug carriers and hyperthermic treatment;

(3) To identify the mechanisms of spontaneous and serum-induced release of solutes from liposomes;

(4) To deliver liposome-encapsulated insulin and insulin fragments to the cytoplasm of adipocytes with the aim of distinguishing the relative roles of surface receptors and internalization of the hormone.

Non-standard Methods Employed: (a) Preparation of liposomes, as described in refs. 3 and 4. (b) Investigation of liposome-cell interactions using the fluorescence-activated cell sorter, as described in refs. 3 and 4. (c) Dynamic measurement of leakage from liposomes as a function of temperature, using a temperature-scanning fluorescence system devised in our laboratory for the purpose. See ref. 5. (d) Measurements of release of solute from liposomes by "fluorescence self-quenching". See refs. 3-5. (e) Determination of the mobility of vesicles bound to cells, using fluorescence-photobleaching. See ref. 4.

Major Findings:

Objective 1: (a) Bivalent antibody selectively binds DNP-bearing liposomes to TNP-bearing lymphocytes, but the binding does not increase delivery of liposome contents to the cell interior (beyond the amount internalized spontaneously). (b) Endogenous surface IgA on cells of the murine myeloma MOPC 315 can bind liposomes bearing the appropriate hapten (DNP) to the cell surface. However, as in the lymphocyte system, binding does not increase delivery to the cytoplasm. (c) Lipid vesicles containing fluorescent molecules are potentially useful as markers for sparse or low-affinity cell-surface determinants. They can be made to contain many fluorophore molecules, thus amplifying the signal. They give very low non-specific background since the fluorophore is sequestered and appropriate lipids are not very "sticky". (d) Liposomes are removed from the circulation of a mouse much faster if the mouse carries a myeloma secreting antibody to a hapten on the liposome.

Objective 2: (a) Small unilamellar vesicles of 3:1 dipalmitoylphosphatidylcholine - distearoylphospatidylcholine release their contents very slowly at 37°C, much faster at 41 - 46°C. Such "custom-designed" liposomes might be useful to achieve high drug concentrations selectively in local areas of hyperthermic treatment, for example, in the treatment of tumors. (b) The temperature-dependence of the release can be enhanced by increasing the rate of temperature change, by using multilamellar vesicles in place of the unilamellar ones, and most markedly, by the presence of serum in the medium.

Ratios of greater than 100:1 can be obtained for release (of a fluorescent marker) at 43 and at 37°.

Objective 3: (a) Release of carboxyfluorescein from small unilamellar vesicles takes place by "leakage", not by an all-or-nothing "rupture" of the vesicle. (b) The rate constant for leakage increases in inverse proportion to the hydrogen ion concentration of the medium. Carboxyfluorescein is a weak anion, more lipid-soluble at low pH. These findings suggest that the species crossing the liposome bilayer is a less-charged species than the trivalent anion prevalent at neutral pH. (c) Liposomes of dioleoyl lecithin leak their contents and form structures with a characteristic appearance in negative-staining electron microscopy when allowed to interact with HDL or LDL. The interaction is faster and more pronounced with isolated HDL apolipoprotein than with the whole lipoprotein particle. (d) Liposomes bearing the DNP-hapten can be made to release carboxyfluorescein in the presence of complement and IgG anti-TNP. Fluorescence self-quenching provides the most sensitive technique available for continuously monitoring such processes. (e) Cholesterol-containing liposomes can pass intact, and without releasing much of their contents, from the peritoneum to the bloodstream of a mouse. This finding is potentially important for possible clinical instillations of liposomes I.P.

Objectives 4: Preliminary experiments only.

Significance to Biomedical Research and the Program of the Institute: The first three objectives listed clearly relate to the possibilities for using liposomes in tumor therapy. A major barrier to such efforts has been the difficulty of directing liposomes to particular cells or anatomical sites. The studies of antibody-mediated "targeting" suggest a way to achieve selectivity but also demonstrate an additional problem: how to get the liposome and its contents into the cell after binding. The studies of synergism between liposomes and hyperthermia indicate a promising new way to achieve selective delivery. Use of the liposome as a hapten-carrier for analysis and sorting of cell populations may be useful in a number of areas of cellular and tumor immunology.

Proposed Course: Each of the 5 objectives specified earlier will be pursued. In the case of the hyperthermic system, emphasis is now shifting from in vitro to in vivo approaches and also to theoretical analysis of the pharmacokinetics. Studies of antibody-mediated "targeting" are now being extended to include assays of the physiological effects of delivered drugs in vitro, as well as the distribution of fluorescence markers.

Publications:

Blumenthal, R., Weinstein, J.N., and Henkart, P.: Lipid model membrane studies of immune cytotoxic mechanisms. In Miller, M.W., and Shamoo, E.A. (Eds.): Adv. in Experimental Medicine and Biology. New York, Plenum, 1977, pp. 495-509.

Pagano, R., and Weinstein, J.N.: Interactions between lipid vesicles and mammalian cells. Ann. Rev. Biophys. and Bioeng., 1978, pp. 435-468.

Blumenthal, R., Weinstein, J.N., Sharrow, S.O., and Henkart, P.: Liposome-lymphocyte interaction: Saturable sites for transfer and intracellular release of liposome contents. Proc. Natl. Acad. Sci. USA 74: 5603-5607, 1977.

Weinstein, J.N., Blumenthal, R., Sharrow, S.O., and Henkart, P.A.: Antibody-mediated targeting of liposomes: Binding to lymphocytes does not ensure incorporation of vesicle contents into the cells. Biochim. Biophys. Acta, In press.

Yatvin, M.B., Weinstein, J.N., Dennis, W.H., and Blumenthal, R.: Use of Hyperthermia to Promote Selective Local Release of Drugs from Liposomes. In Caldwell, W.L., and Durant, R.E. (Eds.): Conference on Clinical Prospects for Hyperthermia. Wisconsin, Madison Press, 1978, In press.

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